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THÈSE

Pour obtenir le grade de :
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Compréhension de la dynamique spatiale et temporelle de populations d'holothuries à La Réunion : facteurs génétiques, alimentaires et environnementaux

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Soutenue le 23 juin 2023 à l'Université de La Réunion devant la commission d'examen composée de :

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Avant-propos

La première référence hypothétique aux holothuries daterait de 450 av. J.-C. par le poète comique grec Épicharme. Par la suite, en 350 av. J.-C., Aristote mentionnait « ὀλοθύριου » dans *History of Animal*, pouvant se traduire par « holothurion », néanmoins la véracité de l'identification est remise en cause. Guillaume Rondelet a fourni la première illustration connue dans *Libri de piscibus marinis* en 1558, en conservant le nom d'holothurie malgré l'erreur probable d'identification par Aristote. Précisément 200 ans plus tard, Carl von Linné, dans ses éditions *Systema Naturae*, classa les 'Holothuria' au sein des mollusques. Jean-Baptiste de Lamarck proposa en 1801 de regrouper les étoiles de mer, les oursins, les ophiures et les holothuries sous le terme « Radiaires Échinodermés ». Durant le 19^{ème} et 20^{ème} siècle, la description d'espèces d'holothuries a rapidement évolué grâce, notamment, à la contribution de Johan Théel, Ernst Haeckel, Alexey Smirnov, Hubert Ludwig, Johann von Brandt, Karl Pearson ou encore Guilielmus Jaeger. Récemment, une large étude génétique menée par Allison Miller en 2017, a profondément remodelé la classification des Holothuries, répartie en 1777 espèces à présent, plusieurs espèces étant nouvellement décrites chaque année.

En parallèle des casse-têtes taxonomiques, la première utilisation des holothuries par l'Homme, pour l'alimentation et la médecine traditionnelle, daterait de la dynastie Ming. La pharmacopée *Bencao gangmu*, publiée en 1593 par Li Shizhen, mentionnait le potentiel des holothuries comme tonique. Depuis, une soixante-dizaine d'espèces sont exploitées de manière commerciale, majoritairement dans les pays asiatiques, correspondant à environ 10 000 t d'holothuries séchées, soit près de 200 millions d'individus extraits des écosystèmes marins chaque année. Par conséquent, sept espèces possédant une forte valeur commerciale sont actuellement classées « en danger » sur la liste rouge de l'Union International pour la Conservation de la Nature, et neuf autres sont classées comme « vulnérable ».

L'approfondissement des connaissances générales sur l'écologie des holothuries répond donc à un besoin urgent face à l'appauvrissement mondial des stocks, afin de mettre en place un large éventail de solutions viables et pérennes pour préserver ces espèces essentielles à la fois pour l'Homme et pour le fonctionnement des écosystèmes marins où elles occupent une place prépondérante.

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<https://www.iucnredlist.org/fr/search?taxonomies=180143&searchType=species>

Résumé

Les récifs coralliens de La Réunion comptent environ 40 espèces d'holothuries dont trois (*Holothuria atra*, *Holothuria leucospilota* et *Stichopus chloronotus*) sont dispersées, avec des densités très élevées (> 1 ind.m²) par endroits. L'objectif de l'étude était de comprendre les facteurs génétiques, alimentaires et environnementaux à l'origine de cette distribution hétérogène dans l'espace et le temps. L'échantillonnage s'est déroulé sur les récifs de l'Hermitage/La Saline et de l'Étang-Salé et s'est étendu de l'hiver 2019 à l'hiver 2021. Dans un premier temps, les individus ont été génotypés à l'aide de marqueurs microsatellites spécifiques. La richesse clonale de *H. atra* et *H. leucospilota* était maximale (R = 1), impliquant un mode de reproduction principal de type sexué. La structure génétique des différentes populations de *H. leucospilota* à travers le récif de l'Hermitage/La Saline était homogène, probablement en raison de flux larvaires réciproques. La richesse clonale de *S. chloronotus* était plus faible (R = 0,24), induisant un rôle prépondérant de la reproduction asexuée pour cette espèce, également démontré par une faible connectivité à l'échelle intra-récifale uniquement. Dans un second temps, l'évolution spatio-temporelle de la densité, de saisons en décennies, a montré que les populations de *H. leucospilota* semblaient désormais stables, à densités élevées, alors que celles d'*H. atra* et de *S. chloronotus* ont diminué depuis deux ans. Paradoxalement, peu de corrélations ont été trouvées entre caractéristiques sédimentaires et densité de *S. chloronotus* et *H. leucospilota*, tandis que *H. atra* semblait liée à des caractéristiques sédimentaires anthropisées. Dans un troisième temps, les analyses sur le comportement alimentaire ont révélé que le taux d'ingestion et la vitesse de déplacement étaient les plus élevés chez *S. chloronotus*, suggérant que cet espèce ingèrait plus de sable pour se nourrir, en couvrant une plus grande surface pour satisfaire ses besoins nutritifs. Cette espèce semble donc être spécialiste alors que les deux autres peuvent être considérées plutôt comme généralistes. Puis, les analyses de métabarcoding ont permis d'identifier plus de 29 000 ASVs bactériens dans le régime alimentaire des holothuries. Les fractions ingérées et assimilées étaient très variables selon les espèces, les saisons et les sites. La structure des communautés de bactéries benthiques a montré des variations spatio-temporelles significatives, sans patron particulier, les phyla dominants étant Planctomycetota, Pseudomonadota et Cyanobacteria. Les Pirellulales ont été les plus assimilées par les trois espèces. Les Shingomonadales et les Spirochaetales ont été principalement assimilées par *H. atra*, les Spirochaetales et les Chloroplastes par *H. leucospilota*, et les Sphingomonadales et les Rhodobacterales par *S. chloronotus*. La distribution hétérogène des holothuries à La Réunion ne semble donc pas liée avec la distribution variable des bactéries benthiques. Dans un dernier temps, deux nouveaux biomarqueurs liés aux dommages oxydatifs des protéines (l'activité du protéasome et le niveau des produits finaux oxydatifs avancés) ont été comparés à l'acétylcholinestérase dans les tissus des holothuries. Ils ont révélé un stress physiologique le plus élevé chez *H. leucospilota*. Cependant, celui-ci pourrait être considéré comme relativement faible car les niveaux d'acétylcholinestérase étaient plus élevés qu'en 2005, signifiant une possible diminution de la pollution du récif par les pesticides depuis cette date. Une quantification directe des pesticides reste nécessaire. *Holothuria leucospilota* est à la fois sensible et résistante, capable de présenter une large gamme de valeurs pour un biomarqueur, mais possède des populations prospères. L'espèce *H. leucospilota* pourrait être considérées comme une indicatrice de la pollution par les pesticides. En conclusion, la distribution inégale de *S. chloronotus* pourrait s'expliquer par la reproduction asexuée plutôt que par la qualité de l'alimentation bactérienne. La distribution de *H. atra* semble en lien avec des marqueurs de pressions anthropiques. Enfin, celle de *H. leucospilota* n'a pu être liée à aucun patron particulier de communautés bactériennes, ni à la reproduction, ni à aucun facteur environnemental évalué dans cette étude. De futures études devraient se concentrer sur le phénomène d'attraction conspécifique et sur les patrons de recrutement de ces espèces. Enfin, d'autres fractions du régime alimentaire des holothuries, telles que les microalgues ou les bactéries de la colonne d'eau, pourraient être explorées.

Mot-clés : Holothuries ; Alimentation ; Métabarcoding ; Ecotoxicologie ; Ecologie des populations ; Structure génétique

Abstract

Coral reefs at Reunion Island count about 40 species of sea cucumbers, three of them (*Holothuria atra*, *Holothuria leucospilota* and *Stichopus chloronotus*) being patchily distributed and reaching very high densities ($> 1 \text{ ind.m}^2$) at some locations. The aim of this study was to understand genetic, feeding and environmental factors driving this heterogeneous distribution, over space and time. Sampling had been realised in the Hermitage/La Saline and Etang-Salé reefs, from Winter 2019 to Winter 2021, along a North-South gradient. Firstly, individuals were genotyped using microsatellite markers. Clonal richness of *H. atra* and *H. leucospilota* were maximal ($R= 1$), meaning that sexual reproduction was the main mode of reproduction used. Genetic structure of scattered *H. leucospilota* populations were similar, probably due to larval fluxes. Clonal richness of *S. chloronotus* was lower ($R= 0.24$), meaning that asexual reproduction dominated for this species. For this species a mild connectivity was found at the intra-reef scale, whereas inter-reef connexions were not detected. Secondly, spatio-temporal monitoring for density, from seasons to decades, show that populations of *H. leucospilota* have reached high densities and seem to be stable now, whereas the one of *H. atra* have declined and those of *S. chloronotus* have drastically decreased for two decades. Paradoxically, new correlations were found between sedimentary characteristics and *S. chloronotus* and *H. leucospilota* density, while *H. atra* density seems related to sediments exhibiting anthropogenic signature. Thirdly, concerning the feeding behaviour, the ingestion rate and speed of *S. chloronotus* were the highest, suggesting that this species needs more sand ingested and forage over a greater surface to fulfill its daily nutritive requirements, compared to the other two species. Consequently, *S. chloronotus* species appears to be a specialist while the others can be rather considered as generalists. Then, metabarcoding analyses have identified more than 29,000 ASV of bacteria in the diet of sea cucumbers. Benthic bacteria communities showed significant spatio-temporal variations, without any general trend, with dominant phyla of Planctomycetota, Pseudomonadota and Cyanobacteria. Ingested and assimilated fractions were highly variable among species, seasons and sites. Pirellulales were the most assimilated by the three species. Shingomonadales and Spirochaetales orders were mostly assimilated by *H. atra*, Spirochaetales and Chloroplasts by *H. leucospilota*, and Shingomonadales and Rhodobacterales by *S. chloronotus*. The patchy distribution of sea cucumbers in Reunion Island seems not related to the variable distribution of benthic bacteria. Lastly, two new biomarkers linked to oxidative protein damage (activity of the proteasome and advanced oxidative end products level) were compared to acetylcholinesterase biomarker in sea cucumber tissues. They revealed that *H. leucospilota* had the highest physiological stress in the reef. However, the latter could be considered as low because the acetylcholinesterase levels were higher than in 2005, possibly meaning a decreasing of the reef pollution by pesticides since then. Direct pesticide quantification is still needed. *Holothuria leucospilota* is both sensitive and resistant, able to register a large range of biomarker values but with thriving populations. Consequently, *H. leucospilota* species could be considered for indication of pollution by pesticides. In conclusion, patchy distribution of *S. chloronotus* could be explain by asexual reproduction more than by bacterial food quality. The distribution of *H. atra* seem related to anthropogenic disturbances. Finally, *H. leucospilota* distribution could not be link to any bacterial community pattern or reproduction or environmental factors assessed in this study. Further studies should focus on conspecific attraction and on the other fractions of the diet of sea cucumbers such as microalga or bacteria from the water column.

Keywords: Sea cucumbers ; Feeding ; Metabarcoding ; Ecotoxicology ; Population Ecology ; Genetic structure.

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Liste des productions scientifiques

Articles produits et acceptés dans le cadre de cette thèse :

- PIERRAT, J., GÉLIN, P., MAGALON, H. & OURY, N. (2020) Isolation and characterization of 20 microsatellite loci from a commercial tropical sea cucumber, *Actinopyga echinites* (Echinodermata, Holothuroidea) in Microsatellite records for volume 12, issue 2. *Conservation Genetics Resources* **12**, 337–351.
- PIERRAT, J., BÉDIER, A., Eeckhaut, I., MAGALON, H. & FROUIN, P. (2022a) Sophistication in a seemingly simple creature: a review of wild holothurian nutrition in marine ecosystems. *Biological Reviews* **97**, 273–298.
- PIERRAT, J., LIBAUD, N., MAGALON, H. & OURY, N. (2022b) Isolation and characterization of 24 microsatellite loci from one of the most widespread sea cucumber *Holothuria (Merthensiothuria) leucospilota* (Echinodermata, Holothuroidea). *Conservation Genetics Resources* **97**, 1–4.
- PIERRAT, J., MAGALON, H., LIBAUD, N. & OURY, N. (2022c) Isolation and characterization of 21 microsatellite loci from the sea cucumber *Holothuria (Halodeima) atra* (Echinodermata, Holothuroidea) reveal low asexual propagation through time in Reunion Island (southwestern Indian Ocean). *Molecular Biology Reports*, 1–8.
- PIERRAT, J., OURY, N., FROUIN, P. & MAGALON, H. Sex or fission? Genetics highlights differences in reproductive strategies of two sympatric fissiparous sea cucumber species in Reunion Island (southwestern Indian Ocean). *Diversity* **15** (670).

Articles produits et soumis en cours de révision dans le cadre de cette thèse :

- PIERRAT, J., FORGET-LERAY, J., BOURDON, E., GIUSTI, N. & FROUIN, P. Sensitive and resistant to pollution? Innovative combination of multi-biomarkers reveals sea cucumbers as potent bioindicators of pollution. *Marine Pollution Bulletin*.
- PIERRAT, J., MONTGOMERY, E.M., PEARCE, C.M., ECKHAUT, I. & FROUIN, P. Nutrition and digestive physiology of Echinozoa. *Elsevier*. pp 141 (**Book chapter**)
- PIERRAT, J., URBISTONDOY, L., MODI, A., VIRAMOUTOU, B. & FROUIN, P. What drives the patchy distribution of tropical sea cucumbers? A multispecific monitoring study. *Journal of Limnology & Oceanography*

Articles en cours de préparation :

- PIERRAT, J., MAGALON, H., NOEL, C., & FROUIN, P. The natural diet of tropical sea cucumbers: variability of bacteria communities in sediments and digestive tract.
- PIERRAT, J., FORGET-LERAY, J., GIUSTI, N. & FROUIN, P. Stressed or relaxed? Monitoring of spatio-temporal stress due to the exposure of xenobiotics in wild sea cucumber populations using glutathione-S-transferase.

Présentation orale :

FROUIN, P., PIERRAT, J., LERAY-FORGET, J., & BOURDON, E. Wild sea cucumber stress: what do enzymatic activities and oxidation protein products tell us about three tropical species (*Holothuria atra*, *H. leucospilota* and *Stichopus chloronotus*). Sea cucumber aquaculture: new challenges. Station Marine de Concarneau. 03–04.10.2022

Vulgarisations scientifiques :

Fêtes de la science (2019) Stand avec aquarium d'holothuries et poster sur la découverte des holothuries et leurs rôles dans le lagon, Université de La Réunion.

Fêtes de la science (2020) Événement en ligne. Les réponses à vos questions sur les concombres de mer. Vidéo YouTube : <https://www.youtube.com/watch?v=K84kvEQNrLI>

MT180 (2021) Participation à la finale régionale de Ma Thèse en 180 secondes. Vidéo YouTube : <https://www.youtube.com/watch?v=ysWhwHeSOys>

Nuit des chercheurs (2021) Soirée diapositives à la découverte des mystères du lagon.

Articles de presse :

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Tables des matières

Introduction	1
Objectifs de l'étude	3
Chapitre 1 –	5
Contexte général de l'étude	5
1. Les holothuries	7
Les holothuries un taxon très diversifié adapté à des nombreux habitats	7
Les holothuries : modes d'alimentation.....	8
Les holothuries dépositives : rôles écologiques multiples.....	9
Les holothuries et l'Homme : une relation ancienne mais menacée.....	12
2. La Réunion : une île tropicale jeune et anthropisée.....	13
Partie I -	17
Populations d'holothuries à forte densité : facteurs d'agrégation	17
Chapitre 2 –	19
Structure génétique des populations d'holothuries : importance des modes de reproduction sexuée et asexuée	19
Isolation and characterization of 24 microsatellite loci from one of the most widespread sea cucumber <i>Holothuria (Mertensiothuria) leucospilota</i> (Echinodermata, Holothuroidea)	23
Statements & Declarations.....	24
Funding.....	24
Competing Interests	25
Author Contributions.....	25
Data Availability.....	25
Acknowledgements	25
Isolation and characterization of 21 microsatellite loci for the sea cucumber <i>Holothuria (Halodeima) atra</i> (Echinodermata, Holothuroidea) reveal low asexual propagation through time in Reunion Island (southwestern Indian Ocean).....	28
Abstract	28

I. Background.....	28
II. Methods and Results.....	28
III. Conclusions.....	29
Keywords.....	29
Acknowledgements.....	29
Introduction.....	29
Materials and Methods.....	31
Sampling design.....	31
Microsatellite library development, primer selection and genotyping.....	31
Data analysis.....	32
Clonal propagation.....	33
Genetic diversity.....	33
Population structure.....	33
Cross-amplification testing.....	34
Results and Discussion.....	34
Clonal propagation.....	37
Genetic diversity.....	38
Population structure.....	38
Cross-amplification.....	39
Statements & Declarations.....	41
Funding.....	41
Competing interests.....	41
Authors' contributions.....	41
Ethics approval.....	41
Availability of data and material.....	41
Supplementary material.....	42
Sex or fission? Genetics highlight differences in reproductive strategies of two sympatric fissiparous sea cucumber species in Reunion Island (southwestern Indian Ocean).....	45
Abstract.....	45
Graphical Abstract.....	46
Keywords.....	46
Introduction.....	47
Materials and Methods.....	49

Sampling design.....	49
Spatial sampling.....	49
Temporal sampling of both species.....	50
Laboratory steps.....	51
Data analyses.....	52
Clonal identification and propagation.....	52
Genetic diversity.....	53
Population structure and differentiation	53
Results	54
MLG and clone identification	54
Clonal diversity of <i>Holothuria leucospilota</i>	54
Clonal diversity of <i>Stichopus chloronotus</i>	56
Clonal propagation of <i>Stichopus chloronotus</i> through space and time.....	58
Population structure and differentiation	59
Discussion.....	63
Importance of the sexual reproduction for <i>Holothuria leucospilota</i>	63
Importance of asexual reproduction for <i>Stichopus chloronotus</i>	65
Differences in reproductive strategies in two sympatric sea cucumber species	66
Conclusion	68
Statements & Declarations.....	68
Funding.....	68
Competing Interests	68
Author Contributions.....	69
Ethics approval	69
Acknowledgements.....	69
Supplementary material.....	70
Chapitre 3 –	77
Variation spatio-temporelle des populations d’holothuries en lien avec leur environnement	77
What drives the patchy distribution of tropical sea cucumbers? A multispecific monitoring study	81
Abstract	81
Keywords	82

Introduction.....	82
Materials & Methods.....	84
Study area and species studies.....	84
Inter-site variability: back-reef gradient.....	85
Intra-site variability: shore to ocean gradient.....	85
Sampling design.....	86
Population metrics	86
Sedimentary factors	86
Feeding features.....	87
Analyses of sediment and faeces samples	87
Statistical analyses.....	89
Population, sedimentary and feeding parameters.....	89
Sedimentary factors driving sea cucumber populations.....	89
Density over long-term monitoring.....	90
Results	91
Population features.....	91
Inter-site analyses: back-reef gradient.....	91
Intra-site variability: shore to ocean gradient for community.....	92
Sedimentary patterns.....	93
Motility and feeding patterns.....	98
Sedimentary factors influencing sea cucumber densities.....	99
Long-term dynamics for density.....	101
Back-reef gradient	101
Shore to ocean gradient at PLA.....	101
Discussion	103
Patchy distribution related to sedimentary factors in an anthropogenic context..	103
Multiseasonal monitoring highlights three distinct patterns of temporal dynamics of sea cucumber species.....	106
Feeding behaviour evidences generalist vs specialist species	109
Conclusion	110
Acknowledgment.....	110
Funding	110
Supplementary Material.....	111
Partie II –	117

Alimentation et stress des holothuries dans un environnement pollué..... 117

Chapitre 4 –119

Bactéries benthiques dans le régime alimentaire de trois espèces d'holothuries : processus d'ingestion, de sélection et d'assimilation119

Sophistication in a seemingly simple creature: a review of wild holothurian nutrition in marine ecosystems.....	123
Abstract	123
I. Introduction.....	124
II. Morphological and physiological aspects.....	127
(1) Tentacle structure and movements during ingestion.....	127
(2) Food-capture mechanism	129
(a) Food particle adhesion	130
(b) Food particle ensnarement.....	134
(3) Digestive and assimilation processes in holothurians	134
(a) Digestive tract morphology.....	134
(b) Cell and enzymatic activities	136
(c) Obtaining nutrients through cloacal water retention	137
III. HOLOTHURIAN FEEDING BEHAVIOUR.....	138
(1) Deposit- or suspension-feeding	138
(2) Daily burrowing cycle	139
(3) Selectivity by deposit-feeding holothurians	141
(a) Size selection of particles	146
(b) Selection of organically rich particles	147
(c) Patch selectivity.....	148
IV. THE SEDIMENT INGESTED BY HOLOTHURIANS: A COMPLEX FOOD SOURCE	149
(1) The living fractions	154
(a) Bacteria	154
(b) Photosynthetic organisms.....	156
(c) Meiofauna	158
(2) Non-living fractions	160
(a) Detrital matter.....	160

(b) Minerals	162
(c) Plastic particles	163
V. CONCLUSIONS	167
VI. ACKNOWLEDGMENTS	168
The natural diet of tropical sea cucumbers: variability of bacteria communities in sediments and digestive tract	169
Keywords	169
Introduction.....	169
Materials & Methods.....	172
Study area.....	172
Sampling design.....	173
DNA extraction and amplification	174
Bioinformatic process.....	175
Statistical analyses.....	175
Results	179
Raw data primary analyses.....	179
‘Available resources’: spatio-temporal benthic bacteria profiles in Reunion Island reefs.....	180
Spatio-temporal variability of benthic bacteria ingested by sea cucumbers	183
Spatio-temporal variability of benthic bacteria assimilated by sea cucumbers	187
Discussion	191
Spatio-temporal variability of benthic bacteria in Reunion Island reefs and relation to the patchy distribution of sea cucumbers	191
Implication of benthic bacteria in the nutritional items of sea cucumbers: does any selection occur?.....	192
Whole snapshot of the diet of sea cucumbers: other resources involved.....	194
Conclusion	195
Acknowledgment.....	196
Funding	196
Supplementary Material.....	197
Chapitre 5 –	223
Stress physiologique chez les populations d’holothuries en réponse à la pollution anthropique	223

Sensitive and resistant to pollution? Innovative combination of multi-biomarkers reveals sea cucumbers as potent bioindicators of pollution	227
Abstract	227
Keywords	228
Introduction.....	228
Materials and Methods	232
Study area.....	232
Sampling design.....	232
Biomarker assays.....	234
(i) Oxidative protein damage.....	234
(ii) Chymotrypsin-like activity of proteasome	235
(iii) Acetylcholinesterase enzymatic activity	235
Population and environmental data.....	236
Statistical analysis.....	236
Results	237
Spatio-temporal variability for individual metrics and ecological variables	237
Biomarker patterns.....	238
(ii) Specific variations.....	241
(iii) Relation between population parameters and biomarkers	242
Discussion	243
Validation of the use of oxidative protein damage biomarkers as tool for monitoring organic pollution.....	243
Specific, spatio-seasonal and pluriannual monitoring of neural response to toxicity and oxidative protein damage highlight the sensitivity of sea cucumbers to organic pollution	243
Healthy sea cucumber populations of Reunion Island reveal their resistance to organic pollution and their bioindication ability	246
Recommendations for future multi-biomarkers analyses on sea cucumbers	247
Conclusion	248
Acknowledgements	249
Funding	249
Supplementary Material.....	250
Discussion générale.....	255
Conclusion	271
Références.....	273

Liste des figures

Figure 1 : Classification des Holothuroidea, modifiée d'après Miller et al. (2017)	8
Figure 2 : Impacts de l'alimentation des holothuries dépositives sur la chimie de l'eau. (a) Pendant la journée, la dissolution partielle du carbonate de sable augmente la disponibilité du CO_3^{2-} pour la calcification des organismes adjacents (tels que les coraux ou les algues vertes). La production de NH_3 et l'absorption de CO_3^{2-} augmentent l'alcalinité totale (AT), ce qui améliore le rapport AT/CID et réduit donc localement l'effet de l'acidification des océans (AO). (b) Pendant la nuit, la production de NH_3 qui augmente le rapport AT/CID est inférieure à la transformation du CO_3^{2-} en CO_2 , ce qui augmente le carbone organique dissous (CAD) et exacerbe l'effet de l'AO (modifié d'après Purcell <i>et al.</i> , 2016).	12
Figure 3 : Location of Reunion Island in the southwestern Indian Ocean, and of L'Hermitage/La Saline reef in the west coast of Reunion Island.	31
Figure 4 : Results of the assignment tests for <i>Holothuria atra</i> individuals from both seasons. A) Structure plot at $K = 11$ and B) DAPC assignments at $K = 11$	39
Figure 5 : Location of the sites of sampling in each reef.	50
Figure 6 : Spatial and temporal clonal distribution of <i>Stichopus chloronotus</i>	59
Figure 7 : Assignment tests for <i>Holothuria leucospilota</i> individuals from the three seasons ($S_{1\text{cold}}$, $S_{2\text{warm}}$, $S_{3\text{cold}}$) on each site	60
Figure 8 : Assignment tests for <i>Stichopus chloronotus</i> individuals from the three seasons ($S_{1\text{cold}}$, $S_{2\text{warm}}$, $S_{3\text{cold}}$) on each site	62
Figure 9 : Location of Reunion Island in the western Indian Ocean. Location of L'Hermitage/La saline and Étang-Salé reefs on the west coast of Reunion Island, and sampling sites at each reef.	86
Figure 10 : Temporal variability of the density (ind.m ²) and length (cm) of <i>S. chloronotus</i> at different site.	92

Figure 11: Temporal variability of the density (ind.m ²) and length (cm) of <i>H. atra</i> (in blue) and <i>H. leucospilota</i> (in pink) over the shore to ocean gradient at PLA.	93
Figure 12: Substrates cover (in %) across the sites of the study over the seasons of monitoring.	96
Figure 13: Moving speed (cm.h ⁻¹) and ingestion rate (gDW.ind ⁻¹ .d ⁻¹) of <i>H. leucospilota</i> (in pink), <i>H. atra</i> (in blue), <i>S. chloronotus</i> (in green) in different times of the day (08 and 10 am, 02 pm) at different sites.	98
Figure 14: Long term comparison of the density (ind.m ²) of <i>H. leucospilota</i> (in pink), <i>H. atra</i> (in blue) and <i>S. chloronotus</i> at MNS, PAS, TE and ES.	102
Figure 15: Long term comparison of the density (ind.m ²) of <i>H. leucospilota</i> (in pink) and <i>H. atra</i> (in blue) at PLA, and within the gradient sites of PLA (PLA_SHORE, PLA_MID and PLA_OCEAN).	103
Figure 16: Cumulative number of articles on “sea cucumber” for general publications and “bêche-de-mer” for those focusing on fisheries and aquaculture since 1950. Note the different scales for the two axes.	126
Figure 17: Examples of holothurian tentacle morphology. (A) Digitate (unbranched); (B) peltate (slightly branched); (C) pinnate (highly branched); (D) dendritic (ultra-branched)..	128
Figure 18: Fine ultrastructure of the holothurian tentacle. Digitate tentacle of (A) <i>Thyonidium</i> sp. and (B) <i>Holothuria forskali</i> . D, discs; P, Papillae; S, shaft. (C) Profile view of a five-bud papilla. B, buds; (D) Arrangement of cilia and microvilli on bud surface. C, cilium; Mi, microvilli. (E, F) Diagrammatic sections through a whole tentacle (E) and through a two-bud papilla (F). AL, ambulacral lumen; Co, connective tissue layer; EN, epineural nerve plexus; Ep, epidermis; HN, hyponeural nerve plexus; Me, mesothelium; Mu, muscles. Modified from Bouland et al. (1982) and McKenzie (1987).	132
Figure 19: Digestive tract morphology of (A) <i>Holothuria atra</i> and (B) <i>H. tubulosa</i> . BR, brown region of the anterior foregut; Cl, cloaca; CR, calcareous ring; DHP, dorsal haemal plexus; Fg, foregut; G, gonads; Hg, hindgut; LT, longitudinal tube of rete mirabile; MC, main channel of	

dorsal haemal plexus; Mg, midgut; RM: rete mirabile; RS, region of severance; RT, respiratory tree; S₁, sphincter 1; S₂, sphincter 2; S₃, sphincter 3; T, tentacles; TS, transverse sinuses; TV, tentacle vesicles; VP, vesicle of Poli; VS, ventral sinus. Modified from Trefz (1958) and Massin (1978, inspired by (Cuenot, 1948))..... 136

Figure 20: Synthesis of food sources for the seven orders of holothurians. Order colours represent different trophic modes: blue, suspension-feeders (Dendrochirotida); black, deposit-feeders. The number of species per taxon is shown below the order name. The width of arrows corresponds to the proportion of food ingested per source. Colours of food sources vary with food type: green, living fraction; grey, non-living fraction. Tentacle type for each taxon is represented by the drawings (see Fig. 2): digitate (unbranched); peltate (slightly branched); pinnate (highly branched); dendritic (ultra-branched). 166

Figure 21: Location of Reunion Island in the Western Indian Ocean. 1) L’Hermitage/La Saline reef and 2) Étang-Salé reef, in the West coast of Reunion Island. 174

Figure 22: UpSetR visualization of interactions between ASVs within the four compartments. 177

Figure 23: Nutritional items for sea cucumbers, with four external or internal compartments. 177

Figure 24: Spatio-temporal variability of the Shannon diversity and Pielou evenness indexes for benthic bacteria included in ‘available resources’ nutritional item, regarding sites and seasons. Significant differences found with Student t-tests. *P*value are indicated in bold... 181

Figure 25: Spatio-temporal diversity of benthic bacteria included in ‘available resources’ nutritional item. Pie charts show the ‘Order Family’ weighing more than 3%, below this threshold all ‘Order Family’ units have been pooled (white caption). Main colour represents ‘Order Family’ from the same Phylum. Different upper cases in the column « Site » indicate significant spatial difference in the diversity of benthic bacteria (ANOSIMs). Different lower cases indicate significant seasonal variation per site (ANOSIMs). Results of ANOSIMs are shown in **Supplementary material 17** and **18**. 182

Figure 26: Spatio-temporal and species variability of the Shannon diversity and Pielou evenness indexes of benthic bacteria in ‘ingestion’ nutritional item. Significant differences found with Student *t*-tests are indicated: *P*value < 0.05: *; < 0.01: ** and < 0.001: ***. Only significant values among the same species are represented (intraspecific), and between species from the same site (intra site; PLA and TE). *Holothuria leucospilota* in pink; *Holothuria atra* in blue and *Stichopus chloronotus* in green. 185

Figure 27: Spatio-temporal diversity of benthic bacteria involved in the ‘ingestion’ nutritional item for each species. Pie charts show the ‘Order Family’ weighing more than 3%, below this threshold all ‘Order Family’ units have been pooled (white caption). Main colour represents ‘Order Family’ from the same Phylum. Different upper cases of the same colour in the « Species » column indicate significant spatial difference in the diversity of benthic bacteria for each species (ANOSIMs). For each row, different lower cases indicate significant seasonal variation per site and species (ANOSIMs). Results of ANOSIMs are shown in **Supplementary material 22 and 23**. 186

Figure 28: Spatio-temporal and species variability of the Shannon diversity and Pielou evenness indexes for benthic bacteria involved in ‘assimilation’ nutritional item, at all sites and seasons. Significant differences found with Student *t*-tests are indicated: *P*value < 0.05: *; < 0.01: ** and < 0.001: ***. Only significant values among the same species are represented, and between species from the same site (PLA and TE). *Holothuria leucospilota* in pink; *Holothuria atra* in blue and *Stichopus chloronotus* in green. 189

Figure 29: Spatio-temporal diversity of benthic bacteria involved in the ‘assimilation’ nutritional item for each species. Pie charts show the ‘Order Family’ weighing more than 3%, below this threshold all ‘Order Family’ units have been pooled (white caption). Main colour represents ‘Order Family’ belonging to the same Phylum. Different upper cases of the same colour in the « Species » column indicate significant spatial difference in the diversity of benthic bacteria for each species (ANOSIMs). For each row, different lower cases indicate significant seasonal variation per site and species (ANOSIMs). Results of ANOSIMs are shown **Supplementary Materials 30 and 31**. 190

Figure 30: Location of Reunion Island in the western Indian Ocean. Location of L'Hermitage/La saline and Étang-Salé reefs in the west coast of Reunion Island, and the location of the sampling sites at each reef..... 234

Figure 31: Spatial variability of biomarkers over the seven sites of the study. (A) Activity of acetylcholinesterase ($\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$) and (B) LLVY proteasome activity (fluorescence arbitrary units $\text{mg}^{-1} \text{protein}$). 240

Figure 32: Specific variability of biomarkers. (A) Advanced oxidative protein product (chloramine units $\text{mg}^{-1} \text{protein}$). (B) LLVY proteasome activity (fluorescence arbitrary units $\text{mg}^{-1} \text{protein}$). (C) Activity of acetylcholinesterase ($\text{nmol min}^{-1} \text{mg}^{-1} \text{protein}$) in muscles ... 242

Figure 33: Influence des facteurs sur la distribution en agrégation à forte densité des trois espèces d'holothuries dominantes à La Réunion. 256

Liste des tableaux

Table 1: Microsatellite loci developed for <i>Holothuria atra</i> over 44 individuals.....	35
Table 2: Cross-amplification for the 21 microsatellite loci of <i>Holothuria atra</i> on four other <i>Holothuria</i> species: <i>H. fuscogilva</i> , <i>H. leucospilota</i> , <i>H. nobilis</i> and <i>Holothuria</i> sp. type “Pentard”.	40
Table 3: Panels for multiplexing the 9 <i>Stichopus chloronotus</i> microsatellite loci	52
Table 4: Indices of genetic diversity for <i>Holothuria leucospilota</i> populations from Reunion Island	55
Table 5: Indices of genetic diversity and clonal structure for <i>Stichopus chloronotus</i> populations from Reunion Island	57
Table 6: Genetic differentiation of <i>Holothuria leucospilota</i> populations with all individuals kept estimated with Weir and Cockerham’s F_{ST}	61
Table 7: Genetic differentiation of <i>Stichopus chloronotus</i> populations with all individuals kept estimated with Weir and Cockerham’s F_{ST}	63
Table 8: Two-ways PERMANOVA results of sedimentary parameters at the back-reef (MNS, PAS, TE and ES) and shore to ocean (PLA) gradient overs sites and seasons	97
Table 9: Significance of individuals and ecological variables of each model (ANOVA results).	100
Table 10: Ultrastructural composition of the tentacle buds of holothuroids with the nomenclatural terms used in analyses by various authors. The first column describes the corresponding cells observed in podia of echinoderms where a duo glandular adhesive system (adhesion and de-adhesion) has been identified.....	133
Table 11: Synthesis of studies on selective feeding strategies in shallow-water and deep-sea holothurians.	142

Table 12: Food sources recorded as ingested by coastal and deep-sea holothurians..... 150

Table 13: Two-ways PERMANOVA results for density and length of *Holothuria leucospilota* and *Stichopus chloronotus*, organic matter load, fine particle and chlorophyll a over sites and seasons..... 238

Table 14: Significance of individuals and ecological variables of each model (ANOVA results).
..... 239

Liste des supplementary materials

Supplementary material 1: Microsatellite loci developed for <i>Holothuria leucospilota</i> over 24 individuals.....	26
Supplementary material 2: Panels for multiplexing the 21 <i>Holothuria atra</i> microsatellite loci.	42
Supplementary material 3: Distribution of pairwise differences (number of alleles shared) among multi-locus genotypes (MLG). Red arrows show potential antimodes separating MLGs of the same multi-locus lineage (MLL) from those of different MLLs.	43
Supplementary material 4: Results of the assignment tests for <i>Holothuria atra</i> . (A) mean likelihood over the five iterations of the same K, (B) Evanno's ΔK distribution, (C) BIC distribution and (D) plots from K = 2 to K = 12 for Structure (above) and DAPC (below).	44
Supplementary material 5: Summary of the sampling design.....	70
Supplementary material 6: Distribution of pairwise differences (number of alleles shared) among multi-locus genotypes (MLG) for <i>Holothuria leucospilota</i>	71
Supplementary material 7: Number of individuals of <i>Stichopus chloronotus</i> per multi-locus genotypes (MLG) identified. The numbering of MLGs comes from Pirog et al. (2017).....	72
Supplementary material 8: Results of the assignment tests for <i>Holothuria leucospilota</i> . (a) mean likelihood over the five iterations of the same K, (b) Evanno's ΔK distribution, (c) BIC distribution and (d) plots from K = 2 to K = 5 for Structure (above) and DAPC (below).....	73
Supplementary material 9: Results of the assignment tests for <i>Stichopus chloronotus</i> . (a) mean likelihood over the five iterations of the same K, (b) Evanno's ΔK distribution, (c) BIC distribution and (d) plots from K = 2 to K = 5 for Structure (above) and DAPC (below).....	74
Supplementary material 10: Results of the assignment tests for <i>Stichopus chloronotus</i> keeping only one representative per MLG, for each site, all seasons pooled. (a) mean likelihood over	

the five iterations of the same K, (b) Evanno’s ΔK distribution, (c) BIC distribution and (d) plots from K = 2 to K = 5 for Structure (above) and DAPC (below)..... 75

Supplementary material 11: Pearson’s correlation results of environmental variables..... 111

Supplementary material 12: Final model of each species (*H. leucospilota*, *H. atra* and *S. chloronotus*) for back-reef and ocean to shore gradient. 112

Supplementary material 13: Distribution of lengths of the three species monitored during this study..... 113

Supplementary material 14: Spatio-temporal variations of sedimentary factors from the back-reef depression gradient. Values are mean \pm se. Different letters indicate significant difference between seasons or sites (Pvalue < 0.005). Results of Tukey-HSD tests (except for $\Delta^{13}C$ seas seasonal variation where Student Newman-Keuls test was used to reveal the significant difference highlighted by ANOVA). 114

Supplementary material 15: Spatio-temporal variations of sedimentary factors from the shore-to-ocean gradient. Values are mean \pm se. Different letters indicate significant difference between seasons or sites (Pvalue < 0.005). Results of Tukey-HSD tests. 115

Supplementary material 16: Rarefaction curves showing the relation between the number of reads and ASV numbers. 197

Supplementary material 17: Benthic bacteria inter-site variability. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold..... 198

Supplementary material 18: Benthic bacteria intra-site seasonal variability. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold. 199

Supplementary material 19: Inter-site and inter-seasonal variability of benthic bacteria *Pirellulales* *Pirellulaceae* in the ‘available resources’ nutritional item. Results of ANOVA and Tukey HSD. Significant Pvalue are indicated in bold..... 200

Supplementary material 20: Inter-site and inter-seasonal variability of benthic bacteria Chloroplast Chloroplast in the ‘available resources’ nutritional item. Results of ANOVA and Tukey HSD. Significant Pvalue are indicated in bold..... 201

Supplementary material 21: Inter-site and inter-seasonal variability of benthic bacteria Rhodobacterales Rhodobacteraceae in the ‘available resources’ nutritional item. Results of ANOVA and Tukey HSD. Significant Pvalue are indicated in bold..... 202

Supplementary material 22: Inter-site variability of benthic bacteria involved in the ‘ingestion’ nutritional item. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold..... 203

Supplementary material 23: Intra-site seasonal variability of benthic bacteria involved in the ‘ingestion’ nutritional item. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold..... 204

Supplementary material 24: Inter-site and inter-seasonal variability of benthic bacteria Pirellulales Pirellulaceae in the ‘ingestion’ nutritional item. 205

Supplementary material 25: Inter-site and inter-seasonal variability of benthic bacteria Rhodobacterales Rhodobacteraceae in the ‘ingestion’ nutritional item..... 206

Supplementary material 26: Inter-site and inter-seasonal variability of benthic bacteria Sphingomonadales Sphingomonadaceae in the ‘ingestion’ nutritional item..... 207

Supplementary material 27: Contribution of ‘Order Family’ involved in the significant difference between benthic bacteria of ‘available resources’ and ‘ingestion’ items for *S. chloronotus*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated..... 208

Supplementary material 28: Contribution of ‘Order Family’ involved in the significant difference between benthic bacteria of ‘available resources’ and ‘ingestion’ item for *H. atra*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated. 209

Supplementary material 29: Contribution of ‘Order Family’ involved in the significant difference between benthic bacteria of ‘available resources’ and ‘ingestion’ items for *H. leucospilota*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated..... 210

Supplementary material 30: Benthic bacteria involved in the ‘assimilation’ nutritional item inter-site variability. Results of ANOSIM and pairwise ANOSIM for each site. Significant *P*value are indicated in bold..... 213

Supplementary material 31: Intra-site seasonal variability of benthic bacteria involved in the ‘assimilation’ nutrition items. Results of ANOSIM and pairwise ANOSIM for each site. Significant *P*value are indicated in bold. 214

Supplementary material 32: Inter-site and inter-seasonal variability of benthic bacteria *Pirellulales Pirellulaceae* in the ‘assimilation’ nutritional item..... 215

Supplementary material 33: Inter-site and inter-seasonal variability of benthic bacteria *Rhodobacterales Rhodobacteraceae* in the ‘assimilation’ nutritional item. Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold..... 216

Supplementary material 34: Inter-site and inter-seasonal variability of benthic bacteria *Sphingomonadales Sphingomonadaceae* in the ‘assimilation’ nutritional item. Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold..... 217

Supplementary material 35: Contribution of ‘Order Family’ involved in the significant difference between benthic bacteria of ‘ingestion’ and ‘assimilation’ nutritional items for *S. chloronotus*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated..... 218

Supplementary material 36: Contribution of ‘Order Family’ involved in the significant difference between benthic bacteria of ‘ingestion’ and ‘assimilation’ nutritional items for *H. atra*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated..... 220

Supplementary material 37: Contribution of ‘Order Family’ involved in the significant difference between benthic bacteria of ‘ingestion’ and ‘assimilation’ nutritional items for *H. leucospilota*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated..... 221

Supplementary material 38: Final model of each biomarker (AChE, AOPP and LLVY)..... 250

Supplementary material 39: Variability of individuals and ecological variables. (A) Mean density (ind.m² ± SE) of *Holothuria leucospilota* through sites. (B) and (D) Mean of length (cm ± SE) of *H. leucospilota* and *Stichopus chloronotus*, respectively, through sites. (C) Mean density (ind.m² ± SE) of *S. chloronotus* through seasons..... 251

Supplementary material 40: Pearson’s correlation results for AChE, LLVY and AOPP with mean population length and density 252

Supplementary material 41: Correlation between, on the left: length of individuals (cm) and on the right: density of individuals (ind.m²) and (A) & (B) activity of acetylcholine esterase (nmol min⁻¹ mg⁻¹ protein); (C) & (D) LLVY proteasome activity (fluorescence arbitrary units mg⁻¹ protein); (E) & (F) advanced oxidative protein product (chloramine units mg⁻¹ protein). 253

Introduction

Un lien fort existe entre l'Homme et l'Océan. Plus de 60% de la population mondiale réside à moins de 150 km de l'Océan (UICN), qui fournit des services écosystémiques estimés à plus d'un milliard de dollars (UNCTAD, 2021). Il subvient quotidiennement aux besoins alimentaires de 15% de l'humanité (Goulletquer *et al.*, 2012). Depuis plusieurs décennies maintenant les scientifiques alertent sur l'état de l'Océan qui se dégrade à une vitesse alarmante. L'Océan est, en effet, soumis à de nombreuses perturbations, telles que la surpêche (Christensen *et al.*, 2014), diverses formes de pollution (Nissanka & Yapa, 2018; Rochman & Hoellein, 2020; Landrigan *et al.*, 2020), l'acidification (Gaylord *et al.*, 2015), la désoxygénation (Oschlies *et al.*, 2018), l'élévation du niveau de la mer (Jevrejeva *et al.*, 2016) ou encore le réchauffement climatique (Levin & Le Bris, 2015; Wijffels *et al.*, 2016). L'ensemble de ces perturbations engendre des conséquences notables sur les espèces marines, dont les populations déclinent de manière drastique (Essington *et al.*, 2015; Rilov, 2016), avec 37% des espèces de raies et de requins, 36% des espèces de coraux ou encore 28% des espèces de crustacées considérées comme en danger critique d'extinction (UICN, 2023).

Parmi les écosystèmes marins, les récifs coralliens représentent une surface mondiale de 249 713 km² (Burke *et al.*, 2011) et abriteraient selon les dernières estimations près de 830 000 espèces (Fisher *et al.*, 2015). Le coût des services écosystémiques supportés par les récifs coralliens s'élève à 30 billions de dollars par an (Cesar, Burke & Pet-Soede, 2003). Cependant, ils constituent un milieu très fragile. Entre 2009 et 2018, près de 14% des coraux ont disparu à l'échelle mondiale (Souter *et al.*, 2021), entraînant par conséquent une perte des espèces qu'ils abritaient et des services écosystémiques rendus.

Au sein de la faune corallienne, les holothuries occupent une place prépondérante dans l'équilibre des récifs (Purcell *et al.*, 2016) et constituent une ressource essentielle pour l'Homme, notamment par l'apport de ressources alimentaires (Toral-Granda, Lovatelli & Vasconcellos, 2008) et de substances pour la pharmaceutique et la médecine traditionnelle (Janakiram, Mohammed & Rao, 2015; Han, Keesing & Liu, 2016a). En conséquence, la plupart des populations naturelles d'holothuries se sont effondrées (Friedman *et al.*, 2011).

Identifier les facteurs régissant la distribution des espèces et évaluer la dynamique spatio-temporelle des populations est l'une des clés essentielles permettant d'alerter les décideurs afin d'adapter les politiques de protection, préservation, gestion et restauration.

Objectifs de l'étude

L'objectif principal de cette étude est d'approfondir les connaissances sur les holothuries tropicales en identifiant les principaux facteurs responsables de l'agrégation en forte densité de certaines espèces présentes à La Réunion. Il s'agit également d'améliorer la compréhension de la dynamique spatio-temporelle des populations d'holothuries tropicales.

Dans un premier temps, le facteur génétique sera examiné en évaluant l'importance de la reproduction sexuée et asexuée chez les trois espèces d'holothuries présentes en forte densité, par le biais de l'indice de richesse clonale, à l'aide de marqueurs microsatellites spécifiques (**Chapitre 2**).

Puis, l'influence des facteurs sédimentaires sur la dynamique spatio-temporelle des populations d'holothuries à l'échelle saisonnière et décennale sera explorée (**Chapitre 3**).

Les facteurs alimentaires seront considérés par l'analyse des communautés bactériennes présentes dans le sédiment et dans les processus nutritionnels de l'ingestion et de l'assimilation (**Chapitre 4**).

Enfin, parmi les facteurs anthropiques, la pollution des récifs par les pesticides sera étudiée grâce à la caractérisation de deux nouveaux biomarqueurs liés aux dommages oxydatifs des protéines en corrélation avec l'acétylcholinestérase, permettant alors d'appréhender le niveau de stress physiologique chez les trois espèces (**Chapitre 5**).

Cette étude contribuera à l'augmentation des connaissances générales sur l'écologie des holothuries tropicales, dont certains aspects sont encore aujourd'hui peu connus. Une meilleure identification des facteurs influençant la dynamique des populations permettra une meilleure gestion et protection des holothuries à l'échelle locale.

Chapitre 1 –

Contexte général de l'étude



*Plage de Trou d'Eau
au sein du récif de La Saline*

-
24.02.20

1. Les holothuries

Les holothuries un taxon très diversifié adapté à des nombreux habitats

Les holothuries sont une classe d'organismes vivants, plus familièrement connues sous le nom de concombres de mer, faisant partie de l'embranchement des Échinodermes (Echinodermata), au même rang taxonomique que les oursins, les étoiles de mers, les ophiures et les crinoïdes. Il s'agit d'une classe relativement diversifiée, avec à l'heure actuelle, plus de 1777 espèces décrites mondialement (WoRMS, 2023). Une récente étude phylogénique de grande envergure (Miller *et al.*, 2017) a redéfini en profondeur les ordres, établis par Pawson & Fell (1965), au sein de la classe Holothuroidea (de Blainville, 1834), et jusqu'ici largement utilisés dans la littérature scientifique. Pawson & Fell (1965) avait proposé une classification basée sur la morphologie générale du corps, des tentacules et des anneaux calcaires des espèces. Ils avaient ainsi défini cinq ordres : Apodida, Aspidochirotida, Dendrochirotida, Elaspodida et Molpadida. L'ordre des Aspidochirotida s'est révélé être polyphylétique, il a donc été supprimé et les espèces affiliées à cet ordre ont été réparties en trois ordres : Holothuriida, Persiculida (en partie) et Synallactida, portant au nombre de sept les ordres au sein de la classe des Holothuroidea (Miller *et al.*, 2017). Parmi ces ordres, celui des Dendrochirotida est le plus diversifié avec 795 espèces décrites tandis que celui des Persiculida est le moins diversifié avec seulement 39 espèces décrites. Au sein de cette nouvelle répartition des espèces d'holothuries, des clades ont été définis, en congruence avec la présence de certains traits morphologiques partagés par plusieurs ordres. Le clade des Actinopoda réunit l'ensemble des ordres, excepté celui des Apodida, dont les espèces possèdent des podia. Le clade des Pneumonophora rassemble cinq ordres, dont les espèces sont munis d'un arbre respiratoire. Enfin, le clade des Neoholothuriida renseigne sur la présence de deux gonades, une de part et d'autre du mésentère dorsale (Miller *et al.*, 2017, **Figure 1**).

Ces différences morphologiques, couplées au nombre important d'espèces, révèlent une radiation adaptative facilitant la colonisation d'habitats (Sokolova, 1958; Hansen, 1975). Les holothuries possèdent une distribution géographique large, des zones polaires (Post *et al.*,

2017; O’Loughlin, Bardsley & O’Hara, 2020), aux zones tropicales (Uthicke, 1999; Resueño & Angara, 2020) en passant par les zones tempérées (Svetashev *et al.*, 1991; Morgan, 2008). La plus grande partie des espèces est distribuée dans la région Indo-Pacifique tropicale (Conand, 1990). Elles colonisent toutes les profondeurs des zones peu profonde (Lee *et al.*, 2018; Marrugo-Negrete *et al.*, 2021) aux zones abyssales (Iken *et al.*, 2001; Jamieson *et al.*, 2011), décrites comme ‘le royaume des holothuries’ (Billett *et al.*, 2001; Kuhnz *et al.*, 2014). Finalement, les holothuries prospèrent dans plusieurs habitats, tel que les récifs coralliens (Mangion *et al.*, 2004; Wolfe & Davey, 2020), les herbiers marins (Bulteel, Jangoux & Coulon, 1992; Wolkenhauer *et al.*, 2010), les mangroves (Romero & Cabansag, 2014), les zones intertidales (Lowes *et al.*, 2023) et les plaines abyssales (Deming & Colwell, 1982).

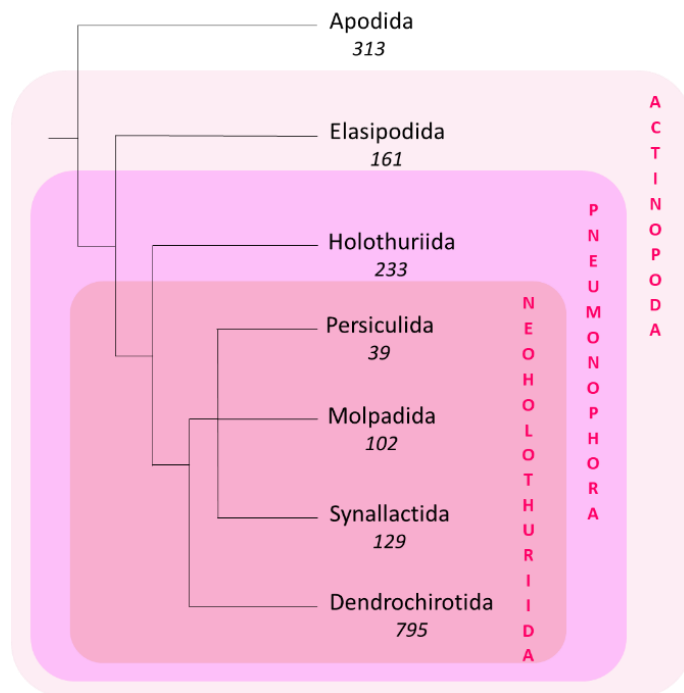


Figure 1 : Classification des Holothuroidea, modifiée d'après Miller et al. (2017)

Les holothuries : modes d'alimentation

Seules les espèces appartenant aux Dendrochirotida sont suspensivores tandis que les autres sont dépositivores. Les holothuries suspensivores vivent dans des conditions hydrodynamiques élevées (Taghon & Jumars, 1984) leur permettant d’intercepter de manière active ou passive les particules (Labarbera, 1984). Les holothuries dépositivores sont

étroitement liées aux compartiments benthiques. Selon Roberts *et al.* (2000), les holothuries dépositives peuvent être subdivisées en trois groupes en fonction de la profondeur de recherche de nourriture : (1) les dépositives épibenthiques se nourrissent à l'interface entre le compartiment benthique, comme le sable, les phanérogames (Cuvillier, 2016), les éponges (Hammond & Wilkinson, 1985) ou les débris coralliens, et la colonne d'eau, par exemple *Holothuria leucospilota* se nourrit sur le sable ou les débris coralliens, (2) les dépositives de subsurface se nourrissent sous la surface des sédiments, comme *Holothuria atra* dont les tentacules atteignent jusqu'à quatre centimètres sous la surface (Taddei, 2006), et (3) les dépositives 'funnel' créent des dépressions dans le sédiment et se nourrissent des particules trouvées dans ces dépressions, par exemple, *Holothuria arenicola* crée des dépressions jusqu'à 10 cm sous le sédiment (Hammond, 1983).

La morphologie, les mécanismes d'alimentation et le régime alimentaire ne sont pas développés dans ce chapitre, puisque développés dans le **Chapitre 4** qui contient une revue publiée sur l'alimentation des holothuries.

Les holothuries dépositives : rôles écologiques multiples

Presque tous les rôles écologiques des holothuries dépositives sont liés à leur comportement nutritionnel, de la recherche de nourriture à la digestion et l'excrétion. Les rôles écologiques des holothuries suspensives sont largement sous-étudiés.

Les holothuries dépositives jouent un rôle écologique important dans les écosystèmes des eaux peu profondes et leurs impacts sur les environnements associés ont été récemment examinés par Purcell *et al.* (2016). Certains auteurs les considèrent comme des espèces clés de voûte (Lawrence *et al.*, 2009; Amaro *et al.*, 2010), c'est-à-dire que leur disparition compromettrait la structure et le fonctionnement des écosystèmes auxquelles elles sont associées. Le principal rôle écologique attribué aux holothuries dépositives est la bioturbation dans les eaux peu profondes (Bakus, 1973; Massin, 1982; Uthicke, 2001; Wolkenhauer *et al.*, 2010) et les environnements profonds (Smith, Matthiopoulos & Priede, 1997; Kaufmann & Smith, 1997). La bioturbation se compose du comportement de fouissage de certaines espèces, qui se cachent dans le sable, et de la quantité considérable de sédiments ingérés

(Bonham & Held, 1963; Ong Che, 1990; Mangion *et al.*, 2004). Ces comportements déstabilisent la surface supérieure des sédiments, ce qui augmente le transfert latéral net de matière organique dissoute de l'eau interstitielle vers la colonne d'eau (Massin, 1982). En outre, la bioturbation oxygène les sédiments (Bakus, 1973; Uthicke, 1999; Mangion *et al.*, 2004), puisque les sédiments en profondeur sont poussés vers la surface et interagissent avec l'oxygène dans la colonne d'eau (Solan *et al.*, 2004). La bioturbation améliore aussi, indirectement, la complexité de la surface des sédiments (bio-rugosité ou rugosité).

Le deuxième rôle écologique principal holothuries dépositives est le recyclage des nutriments. Les principaux produits de sécrétion, dérivés de la digestion et de la reminéralisation des sédiments, sont l'ammonium, de petites quantités de phosphate (Mukai *et al.*, 1989) et des formes dissoutes d'azote et de phosphore organique (Webb, Dupaul & D'elia, 1977). Parmi ces produits de sécrétion, l'ammonium est le plus dominant en termes de quantité libérée de l'extrémité du tube digestif (Uthicke, 2001) vers l'eau de mer environnante (Webb *et al.*, 1977; Mukai *et al.*, 1989). Il a été démontré que ces déchets azotés sont avantageux pour les communautés de macroalgues et de diatomées photoautotrophes dans les sédiments environnants (Uthicke & Klumpp, 1997; Uthicke, 2001). En outre, ils sont utilisés par les herbiers marins (Wolkenhauer *et al.*, 2010), augmentant leur productivité et produisant ainsi plus de nourriture pour les herbivores avoisinants. Les holothuries dépositives améliorent également la productivité d'autres habitats tels que les récifs coralliens (Birkeland, 1988; Uthicke, 1999, 2001; Schneider *et al.*, 2011) ou les forêts de laminaires (Harrold & Pearse, 1989).

Enfin, les holothuries participent à l'équilibre carbonaté des récifs, à une échelle très localisée et où de forte densité peuvent être observée, grâce à une dissolution primaire et secondaire par le tube digestif (**Figure 2**). Les holothuries ont un pH intestinal faible (Bakus, 1973) et Khripounoff & Sibuet (1980) ont suggéré que la diminution de la granulométrie des particules minérales est due à leur processus digestif. Quelques études soutiennent que cette dissolution primaire est une composante importante de la dissolution naturelle du carbonate de calcium (CaCO_3) (Schneider *et al.*, 2011; Plotieau, 2012; Silverman *et al.*, 2012), qui a un impact positif sur les organismes environnants, tels que les coraux. Les ions carbonates (CO_3^{2-}

) libérés lors de la décalcification, par le tube digestif des holothuries, sont disponibles pour l'absorption immédiate par les organismes calcaires adjacents tels que les coraux ou les algues vertes calcaires (Borowitzka & Larkum, 1976 ; De Beer & Larkum, 2001) qui prospèrent dans les récifs où les holothuries sont présentes en fortes densités (Chao, Chen & Alexander, 1993; Lee *et al.*, 2018). La dissolution secondaire modifie la chimie de l'eau de mer (Schneider *et al.*, 2011, 2013; Vidal-Ramirez & Dove, 2016). La dissolution du sable carbonaté dans le tube digestif de l'holothurie entraînerait la production de fèces dont le pH est plus élevé que celui de l'eau environnante (Hammond, 1981; Schneider *et al.*, 2011, 2013), ce qui augmenterait les concentrations totales d'ammoniac dans l'eau de mer (Uthicke & Klumpp, 1998; Uthicke, 2001; Vidal-Ramirez & Dove, 2016). La dissolution du CaCO_3 couplée à l'excrétion d'ammonium augmente l'alcalinité totale (TA) locale de l'eau de mer (Hammond, 1981; Uthicke & Klumpp, 1998; Schneider *et al.*, 2011). Le rapport entre l'alcalinité totale et le carbone inorganique dissous (DIC) permet d'estimer la capacité tampon de l'eau de mer (Wang *et al.*, 2013). Le métabolisme des holothuries (dissolution du sable CaCO_3 , sécrétion d'ammonium) augmente le rapport TA/DIC (Schneider *et al.*, 2013). À l'échelle locale, une population à forte densité pourrait augmenter le rapport TA/DIC et améliorer partiellement la capacité des récifs à réduire le pH de l'eau de mer (Lee *et al.*, 2018) associé à l'augmentation du CO_2 atmosphérique (Stumm & Morgan, 1996). Cette dissolution secondaire, comme la dissolution primaire, a un impact positif sur les coraux, réduisant l'impact de l'acidification des océans sur leur croissance (Schneider *et al.*, 2011, 2013).

Cependant, la quantité moyenne de CaCO_3 dissous est différente entre le jour et la nuit selon les espèces d'holothuries et dépend de leur cycle d'alimentation journalier (voir chapitre 1 et 3; Yamanouchi, 1939, 1956; Mercier, Battaglene & Hamel, 1999). Ainsi, le rôle écologique des holothuries dépositives sur le soutien de la croissance des coraux est débattu. Vidal-Ramirez & Dove (2016) ont travaillé sur *H. atra*, qui se nourrit constamment de jour comme de nuit (Uthicke, 1994) et ont montré que cette espèce a un impact plus important sur le ratio TA/DIC pendant la nuit. Par conséquent, la capacité tampon de cette espèce se produit au mauvais moment du cycle diurne, ce qui entraîne un effet négatif. En outre, les coraux et autres calcificateurs récifaux ont des taux de calcification plus élevés le jour (Dove *et al.*, 2013; Eyre, Andersson & Cyronak, 2014). Vidal-Ramirez & Dove (2016) ont conclu que les espèces

d'holothuries possédant un cycle d'alimentation continue, exacerberont les effets de l'acidification des océans, en éliminant le carbonate de calcium de l'eau de mer et en diminuant la capacité tampon du récif de jour (**Figure 2**).

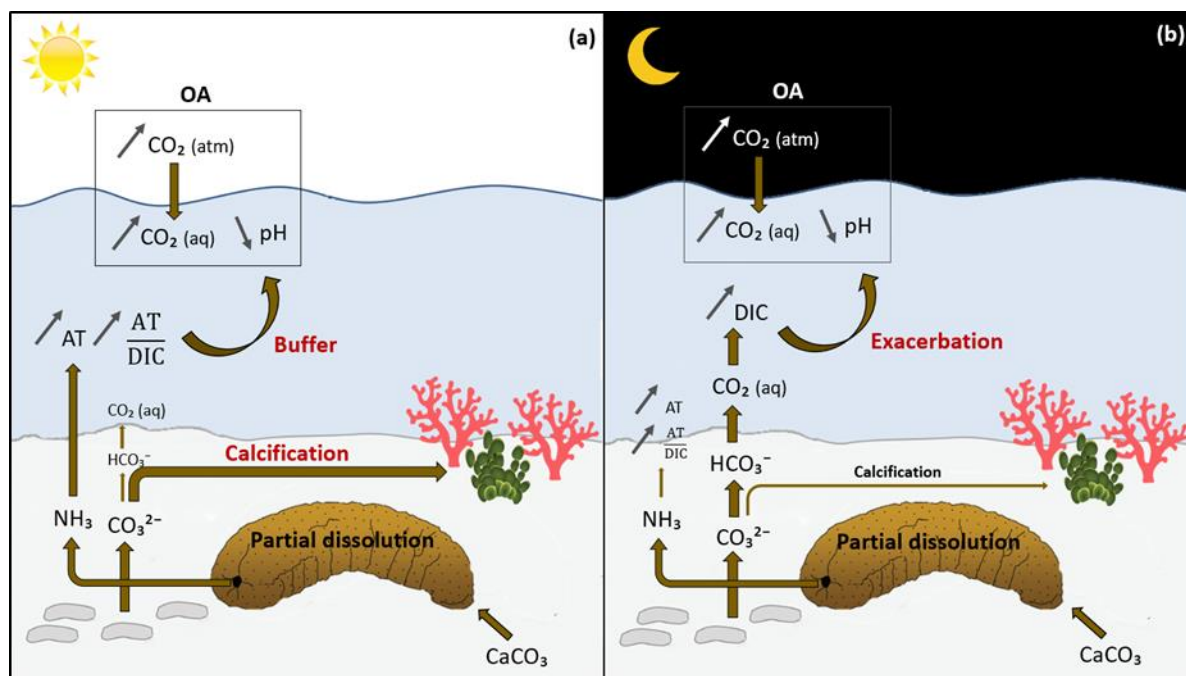


Figure 2: Impacts de l'alimentation des holothuries dépositives sur la chimie de l'eau. **(a)** Pendant la journée, la dissolution partielle du carbonate de sable augmente la disponibilité du CO_3^{2-} pour la calcification des organismes adjacents (tels que les coraux ou les algues vertes). La production de NH_3 et l'absorption de CO_3^{2-} augmentent l'alcalinité totale (AT), ce qui améliore le rapport AT/DIC et réduit donc localement l'effet de l'acidification des océans (AO). **(b)** Pendant la nuit, la production de NH_3 qui augmente le rapport AT/DIC est inférieure à la transformation du CO_3^{2-} en CO_2 , ce qui augmente le carbone organique dissous (CAD) et exacerbe l'effet de l'AO (modifié d'après Purcell *et al.*, 2016).

Les holothuries et l'Homme : une relation ancienne mais menacée

Les holothuries sont exploitées depuis plusieurs centaines d'années par l'Homme. Sur l'ensemble des espèces décrites, approximativement 70 sont pêchées et exploitées, notamment pour la médecine traditionnelle, la consommation alimentaire (plus connu sous le nom de « bêche-de-mer » ou « trepang ») ou en aphrodisiaque (Conand, 2018 ; Toral-Granda, Lovatelli & Vasconcellos, 2008). Certaines espèces sont revendues à prix d'or sur les marchés asiatiques. La majorité des stocks naturels se sont drastiquement appauvris (Friedmann *et al.*, 2011) pour satisfaire la demande asiatique croissante (Rahman, Yusoff &

Arshad, 2015), avec plus de 200 millions d'individus pêchés dans le milieu marin chaque année (Chen, 2003). Les plans de restauration et de gestion de pêche semblent inefficaces quand les stocks sont trop appauvris (Friedmann *et al.*, 2011). Récemment, trois espèces d'holothuries ont été enregistrées dans l'Appendice II de la CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Il s'agit de *Holothuria fuscogilva*, *Holothuria nobilis* et *Holothuria whitmaei* (Di Simone *et al.*, 2019). En outre, sept espèces sont également considérées comme en danger critique d'extinction d'après l'UICN (Union International pour la Conservation de la Nature). En conséquence, le marché mondial se tourne davantage vers des espèces à faible valeur commerciale (Purcell, Samyn & Conand, 2012b). Les pêcheries d'holothuries ont quadruplé durant la dernière décennie (Rahman *et al.*, 2015). L'aquaculture représente une perspective durable pour l'exploitation des holothuries et leur protection dans les milieux naturels. Les processus aquacoles sont aujourd'hui bien développés pour deux espèces *Holothuria scabra* (Hamel *et al.*, 2022) et *Apostichopus japonicus* (Yang, Hamel & Mercier, 2015). Il apparaît alors primordial d'étudier les espèces d'holothuries dans leur environnement naturel, afin de comprendre les facteurs influençant la dynamique spatio-temporelle de leurs populations afin d'obtenir des connaissances fondamentales applicables pour la domestication de nouvelles espèces.

2. La Réunion : une île tropicale jeune et anthropisée

La Réunion est une île française située à l'Est de Madagascar, dans le Sud Ouest de l'océan Indien (21°06'S, 55°31'E). Elle fait partie de l'archipel des Mascareignes, avec Rodrigues et Maurice. De part son histoire géologique et sa localisation géographique, La Réunion présente des caractéristiques importantes. Premièrement, La Réunion est une île volcanique jeune, datant de trois millions d'années (Duncan *et al.*, 1989). Deux volcans, dont l'un est toujours actif (Piton de la Fournaise), participent au relief abrupt et raide de l'île, dont le point culminant s'élève à 3070 m (Piton des Neiges). Le réseau hydrographique de La Réunion est extrêmement dense, avec 24 rivières et des centaines de cours d'eau secondaire. Deuxièmement, La Réunion est une île tropicale sujette à deux saisons marquées. L'été austral, de novembre à avril, est caractérisé par un climat chaud et humide tandis que l'hiver austral, de mai à octobre, est caractérisé par un climat froid et sec (Conand *et al.*, 2007). Lors de la

saison chaude, en été austral, de fortes pluies peuvent survenir ainsi que des cyclones tropicaux d'intensité variée. La combinaison des caractéristiques 'jeune' et 'tropicale' conduit à une asymétrie du climat entre les côtes est et ouest, de part des barrières géographiques élevées. Les précipitations sur la côte est sont plus importantes que sur la côte ouest, car elle est soumise aux alizés océaniques venant des l'est. En conséquence, la côte ouest est d'avantage privilégiée par la population locale et un gradient de densité d'habitant est observé. Le nombre d'habitant ne cesse d'augmenter sur l'île et frôle désormais les 900 000 habitants, inégalement répartis, avec une urbanisation plus prononcée à l'ouest. L'agriculture (38,650 ha de champs), en particulier la culture de la canne à sucre, est l'un des secteurs clés de La Réunion, après le tourisme (DAAF Réunion, 2021). L'agriculture utilise de nombreux pesticides et d'engrais enfin de favoriser le rendement. Près de 200 types de pesticides sont importés à La Réunion (Martin *et al.*, 2013), dont la plupart sont classés en catégories 'N' selon la Commission Européenne, signifiant qu'ils sont « très toxique pour les organismes aquatiques, pouvant provoquer des effets négatifs à long-terme dans l'environnement aquatique ».

La Réunion possède quatre récifs de type frangeant, le récif de l'Hermitage/La Saline, de Saint-Leu, de l'Etang-Salé, tous trois situés sur la côte ouest, et de Saint-Pierre au sud de l'île. Leur superficie représente 18 km². Les récifs situés sur la côte ouest sont sous la gouvernance de la Réserve Naturelle Marine de La Réunion, créée en 2007. Comme La Réunion est une île jeune tropicale et anthropisée, cela entraîne des conséquences sur les récifs, avec des ruissellements et des infiltrations d'eaux terrestres, exacerbés en saison chaude. Des phénomènes de blanchissement corallien, des coulées de boue ou encore l'apport de pesticides dans les récifs sont observés. L'ensemble des descripteurs de l'état de santé du récif en fonction des compartiments récifaux (substrats meubles, durs, assemblages coralliens, colonnes d'eaux) décrivent un état de dégradation avancé du récif.

Les récifs coralliens de La Réunion abritent une quarantaine d'espèces d'holothuries. Trois espèces sont présentes en forte densité (> 1 ind.m²), formant des agrégations inégalement réparties dans les récifs. Il s'agit d'*Holothuria leucospilota*, *Holothuria atra* et *Stichopus chloronotus*, toutes les trois dépositives. Plusieurs études ont caractérisé les

fluctuations spatio-temporelles des populations de ces trois espèces (Conand *et al.*, 1998; Jaquemet, Rousset & Conand, 1999; Conand & Mangion, 2002; Cuvillier, 2016), mais aucune étude à large échelle, ni d'étude sur l'identification des facteurs permettant d'expliquer la répartition hétérogène des populations d'holothuries, n'a été menée. Ces trois espèces ont été décrites comme capable de réaliser à la fois une reproduction sexée et asexée (Dolmatov, 2014).

Partie I -

**Populations
d'holothuries à forte
densité : facteurs
d'agrégation**

Chapitre 2 –

Structure génétique des populations d'holothuries : importance des modes de reproduction sexuée et asexuée



*Individus de Stichopus chloronotus
dans la dépression d'arrière récif du Bassin Pirogue
au sein du récif de L'Etang-Salé*

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19.02.19

Résumé

Les holothuries ont la capacité de se reproduire de deux manières, par voie sexuée via l'émission de gamètes et par voie asexuée via la fission transversale. Le premier mode de reproduction est basé sur la synchronisation de l'émission des gamètes, puis la fécondation externe. Les larves ainsi formées migrent au grès du courant. Les juvéniles se retrouvent donc à distance des adultes, bien qu'un recrutement grégaire puisse exister mais pas encore démontré chez ces trois espèces. Concernant le second mode de reproduction, un individu adulte se scinde en deux par fission transversale et conduit à deux individus au stade adulte identiques génétiquement, sans migration de l'habitat initial. Les individus ainsi scindés se régénèrent au bout de quelques semaines. La reproduction asexuée est recensée chez 16 espèces d'holothuries, soit moins de 1% de l'ensemble des espèces.

A la Réunion plusieurs études ont mis en évidence l'utilisation de la reproduction asexuée pour les trois espèces présentes en forte densité, *H. atra*, *H. leucospilota* et *S. chloronotus*, grâce à des recensements visuels. Ces études sont anciennes et datent de près de 20 ans. Des études récentes se sont focalisées sur la reproduction de *S. chloronotus* à l'aide d'outils génétiques et ont confirmé l'utilisation de la reproduction asexuée chez cette espèce par l'identification d'individu partageant le même génotype. Aucune étude génétique n'a été menée à La Réunion sur la génétique de *H. atra* et *H. leucospilota*.

Ce chapitre vise, dans un premier temps, à étudier l'utilisation des modes de reproduction par *H. atra* et *H. leucospilota* à La Réunion par le biais d'outil génétique ; puis, dans un second temps, à poursuivre les analyses génétiques menées sur *S. chloronotus*.

Les résultats de ce chapitre sont publiés dans *Conservation Genetics Resource, Molecular Biology Reports et Diversity*.

Isolation and characterization of 24 microsatellite loci from one of the most widespread sea cucumber *Holothuria (Mertensiothuria) leucospilota* (Echinodermata, Holothuroidea)

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Holothuria (Mertensiothuria) leucospilota (Brandt, 1835), commonly called “white thread fish”, is one of the most widespread sea cucumber species in the tropics. Its IUCN status is Least Concern (Conand, Purcell & Gamboa, 2013). However, this status needs to be updated since the last evaluation dates from 2010 and because its population trend is unknown while this species is on the original FAO list of commercial species (Purcell et al. 2012). To evaluate the genetic diversity of *H. leucospilota*, and in fine its population status, we isolated and characterized 24 microsatellite markers.

Twenty-four individuals were sampled (only a piece of tegument) in the reef of Reunion Island (21°06'S, 55°31'E), Southwestern Indian Ocean. Total genomic DNA was extracted using DNeasy Blood & Tissue kit (Qiagen™), following the manufacturer's protocol. To identify sequences containing simple sequence repeat (SSR) motif, DNA from eight individuals were sent at Ecogenics GmbH (Switzerland) for sequencing following an SRR enrichment protocol. Primer pairs were then designed using the pipe 3 of QDD 3.1 (Megléc et al., 2014) for each of the 315 promising SSR candidates. Markers with a QDD design note equal to “A” or “B” were retained. Possible primer-dimers formations were checked using

Multiple Primer Analyzer on the web (ThermoFisher Scientific). Then, 96 markers were selected for amplification test depending on the number of repeats (≥ 7), the product size (≥ 100 bp), the absence of primer-dimer and the primer alignment score to the sequence.

The 96 primer pairs selected were then tested for amplification and polymorphism on the eight individuals previously used. Forward primers were indirectly fluorochrome labelled (6-FAM) by adding a universal 19-bp M13 tail at their 5'-end (5'-CAC GAC GTT GTA AAA CGA C-3'). PCRs were then performed with Veriti™ Thermal Cyclers, in a total volume of 10 μ L with MasterMix Applied 1X (Applied Biosystems), 0.025 μ M of forward primer tagged with the M13 tail, 0.25 μ M of reverse primer, 0.25 μ M of fluorescent dyed M13 tail and ca. 2 $\text{ng}\cdot\mu\text{L}^{-1}$ of genomic DNA. The thermocycling program was the following: 94°C for 5 min + 7 \times (94°C for 30 s, 62°C [-1°C at each cycle] for 30 s, 72°C for 30 s) + 35 \times (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) + 8 \times (94°C for 30 s, 56°C for 30 s, 72°C for 30 s) + 72°C for 5 min. PCR products were genotyped using an ABI3730XL sequencer (Applied Biosystems) at the Plateforme Gentyane (INRAE, Clermont-Ferrand, France). Allelic sizes were determined with GeneMapper 4.0 using an internal size standard (Genescan LIZ-500, Applied Biosystems). Primer pairs were selected for further development when (1) they amplified in at least five individuals, (2) they were polymorphic, (3) they did not amplify multiple fragments, and (4) genotype scoring was deemed reliable.

The characterization of the retained loci was then performed by genotyping all 24 individuals. For each locus (**Supplementary material 1**), the number of alleles (N_a) and the observed and expected heterozygosities (H_o and H_e , respectively) were estimated with FSTAT 2.9.3.2 (Goudet, 2001). Departures from Hardy–Weinberg equilibrium were tested with Genepop 4.7.0 (Rousset, 2008).

Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

JP, NL and NO collected samples and did laboratory steps. JP, HM and NO analysed the data. JP wrote the original draft. JP, HM and NO reviewed and edited the manuscript.

Data Availability

Accession Numbers: Primer sequences are deposited on GenBank with accession numbers from OK085410 to OK085433.

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Supplementary material 1: Microsatellite loci developed for *Holothuria leucospilota* over 24 individuals.

Locus name	Primer sequence (5'-3')	Repeat motif	Size range (bp)	Sample size (N)	Number of alleles (N _A)	H _o	H _e
HI01	F: M13-ATCGTGTTTACAAGCTAGGCG R: AGATGTTGCTAGACCACTGCAT	(AC) ₁₁	239–291	20	16	0,800	0,951
HI02	F: M13-CCGTAAGGCATCGAGTGTG R: ACATTGAGAAGGAAGCTTGA	(AC) ₁₁	130–134	23	4	0,417	0,442
HI03	F: M13-TTTCATTATGTTGCACCCACC R: TGTAAGCACAACCTTGCCTG	(AC) ₁₂	134–156	24	7	0,292	0.762***
HI04	F: M13-CCCAGAAGCTCTGGAACATT R: TGCTATGTAAACTGAAGCCAAA	(AC) ₁₂	170–184	24	15	0,833	0,918
HI05	F: M13-ATTGGCAGGCAAGGAATCTA R: GTCTATGTCGCCTGATGGCT	(AC) ₁₃	166–180	23	9	0,174	0.837***
HI06	F: M13-CGTCACGTTACGAATGGTACTC R: TTGGCGCATTTCTTACAAT	(AC) ₁₃	192–208	22	5	0,527	0,640
HI07	F: M13-AACTGGCTTCAATGACACTACG R: TTGATCGCTTGGTTATTGAGTT	(AC) ₁₃	205–221	24	14	0,850	0,926
HI08	F: M13-AATCTGGTCTGCTTTCAGGA R: AACTGCCTGGGTAAGTCTGT	(AC) ₁₄	126–138	24	9	0,833	0,879
HI09	F: M13-GAATAATCACAAGTTTGACGGC R: TAATCTTGAGAAGCCGGTGT	(AC) ₁₄	145–189	23	10	0,748	0,813
HI10	F: M13-AAACGTCCTCGATTGACAGC R: TCTGCTAGCCAAATTACAGGG	(AC) ₁₄	137–165	24	8	0,758	0,859
HI11	F: M13-GAACTAACAGCCACGATTGG R: CGCATAAACTGTGAAGAAGATCC	(AC) ₁₄	201–215	23	14	0,765	0,905
HI12	F: M13-CAGCACATAGTATACTGCATTCCC R: AAATCCGTCCTGCAAAGAA	(AC) ₁₅	268–278	24	7	0,292	0.610***

HI13	F: M13-CAAGTGTTCCAAACCTGGGCT R: TCTTCGGGAAGTGTAGTTGC	(AC) ₁₇	133–165	22	12	0,772	0,911
HI14	F: M13-TGCAGTGCCATATCCAACAT R: TTCTTTCATCCTCTCGGCAT	(AC) ₁₈	129–149	24	10	0,725	0,841
HI15	F: M13-TCCAAGTATGAGATCCGTCG R: CAGTCCTTGCCGAATGCT	(AC) ₁₉	144–168	24	12	0,917	0,898
HI16	F: M13-TAGAAATCCTTCCGCGTGT R: GATGCCCTCGGATTGTATGT	(AC) ₂₀	200–228	20	10	0,655	0,796
HI17	F: M13-GAATCTTATAATCCCTTGTTCTCA R: TCGATCTAACATATAGAATCGTTGG	(AAC) ₂₃	273–321	22	11	0,800	0,83
HI18	F: M13-CACGAACAGATTTCTTTGTTGTTCC R: TGTGGAAGATCACGGGTAAG	(AACG) ₁₅	132–174	23	16	0,897	0,949
HI19	F: M13-GCCGATTCCTTTGAACATTA R: AATTGGTTGGAAACTGGGAC	(AACT) ₁₀	91–132	23	7	0,722	0,858
HI20	F: M13-CGGGTGCAGAAAGTACCCTA R: GGTTCCTCAACTCCCTGGTCTT	(AATC) ₈	130–174	24	7	0,783	0,825
HI21	F: M13-TGTTTCACGAATGAATGAACG R: GCTTGTAAGCCATTTGTACCTT	(AATG) ₁₇	220–320	23	21	0,809	0,950
HI22	F: M13-TCAGGTGATTAGTAGCTCAGCAAG R: CCAACTTTGAGAAGGAACGG	(ACAG) ₈	143–185	21	11	0,576	0,713
HI23	F: M13-GGTCAAAGAACCTGCAGACA R: CCCGACTCAAGCATTACTTAAA	(ACAG) ₉	238–274	23	10	0,752	0,826
HI24	F: M13-GTTAATACGTCAAGTAACGTAGACTGC R: TTCCTTCTTATTTGGCGAGC	(ACAG) ₉	294–304	22	6	0,664	0,756

Deviation from Hardy–Weinberg equilibrium is indicated: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Isolation and characterization of 21 microsatellite loci for the sea cucumber *Holothuria (Halodeima) atra* (Echinodermata, Holothuroidea) reveal low asexual propagation through time in Reunion Island (southwestern Indian Ocean)

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Abstract

I. Background

Holothuria (Halodeima) atra Jaeger, 1833 is a tropical sea cucumber usually harvested for the “bêche-de-mer” trade market. It has been reported to reproduce both sexually, through gamete spawning, or asexually, through fission. To date, no study has ever investigated clonality, nor genetic connectivity, among its populations, using microsatellite markers.

II. Methods and Results

We isolated the first 21 microsatellite loci specific for *H. atra*, which were then used to investigate clonal diversity, genetic structure and diversity among 44 *H. atra* individuals sampled in Reunion Island (southwestern Indian Ocean), over two seasons. All 21 loci were polymorphic, with number of alleles per locus ranging from 2 to 10. No repetitive multi-locus genotype (MLG) and few clonal lineages (MLL) were found. Observed heterozygosities per locus and season ranged from 0.000 to 0.909, while expected heterozygosities ranged from 0.290 to 0.882. Four loci were at Hardy-Weinberg equilibrium for both seasons, all others

presenting a deficit of heterozygotes in one or both seasons. Meanwhile, no genetic differentiation was detected between seasons, according to assignment tests and global F_{ST} .

III. Conclusions

These results suggest low asexual propagation in this population. These loci represent useful tools to better understand reproductive strategies and population connectivity of *H. atra*, and thus provide relevant knowledge for efficient management.

Keywords *Holothuria atra* • Microsatellite • Sea cucumber • Asexual reproduction • Temporal stability • Population structure

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Introduction

Some species of sea cucumbers are harvested for their dried body wall, commonly known as “bêche-de-mer”, “trepang” or “hai-som”, destined to the Asian market (Rahman & Yusoff, 2017). The harvest of sea cucumbers is considered as a valuable commercial resource in coastal fisheries from many Asian countries, where China represents the biggest market. The strong demand for “bêche-de-mer” has led to an overexploitation and a decline of most populations (Purcell *et al.*, 2012a). In order to preserve species in their natural habitat, it is mandatory to get a better knowledge of the biological parameters of the individuals (e.g. age of maturity, longevity or life cycle), as well as the ecological parameters of the populations (e.g. size and its variations over time, reproductive success).

Commonly called “black sea cucumber” or “lollyfish”, largely distributed in tropical waters, especially in the Indo-west Pacific and living mostly on soft substrates in back reefs and reef flats, the sea cucumber *Holothuria (Halodeima) atra* Jaeger, 1833 is on the original FAO list of commercial species (Purcell *et al.*, 2012a). Individuals are frequently harvested by hand in shallow water, in at least 20 countries (Purcell *et al.*, 2012a). It is a sea cucumber from

the Holothuriida order with a medium sized body (ca. 20 cm). This species can reproduce by sexual (through gamete spawning) and asexual (transversal fission) reproductions (Chao *et al.*, 1993; Uthicke, 1997). Asexual propagation usually occurs in cold season (Chao *et al.*, 1993; Uthicke, 1997) and would be triggered by a synergy of environmental factors such as low tide and increase of temperature (Chao *et al.*, 1993; Uthicke, 1997). Fission rate reaches 18% in Taiwan (Chao *et al.*, 1993), and up to 26% in Australia (Uthicke, 1997).

Few studies have evaluated the genetic connectivity of *H. atra* populations nor the impact of asexual reproduction on them (Uthicke, Conand & Benzie, 2001; Skillings, Bird & Toonen, 2011; Lopez, Eastwood & Drew, 2017). Using six allozymes, populations of Torres Strait (western Pacific) and Reunion Island (southwestern Indian Ocean) were found significantly genetically different (Uthicke *et al.*, 2001). Sequencing mitochondrial COI, populations of *H. atra* were significantly differentiated between archipelagos in the northern central and west Pacific and between sites within an archipelago (Skillings *et al.*, 2011), while all populations in four reefs in Fiji were genetically connected with gene flow moving from east to west (Lopez *et al.*, 2017).

In Reunion Island (southwestern Indian Ocean), individuals of *H. atra* are found in the back-reef depression of the fringing reef of L'Hermitage/La Saline, on the west coast of the island. They live on sand and dead coral rubble. *Holothuria atra* individuals are very abundant in only one site, called "Planch'Alizés", where they reach a high density (between 4 and 6 ind.m⁻²) (Conand, 1995, 2004), and only few individuals are scattered throughout the rest of the reef. This population has been monitored for 30 years (Conand, 1995, 2004). In 2000, visual censuses of individuals undergoing fission indicated that individuals resulting from asexual reproduction reached 15% (Conand, 2004). No study has since evaluated the importance of asexual reproduction in *H. atra* population in Reunion Island.

Here, in order to evaluate more precisely the genetic structure and diversity of *H. atra* population in Reunion Island, we isolated and characterised for the first time 21 microsatellite loci for this species. Then, analyses of clonality were performed to quantify the importance of asexual reproduction in this population. Finally, we performed structuring analyses between

seasons to investigate whether sexual and/or asexual reproductions impact the genetic structure and diversity of this population through time.

Materials and Methods

Sampling design

Sampling was carried out in Planch'Alizés (west coast of Reunion Island; 21°06'S, 55°31'E) in the fringing reef of L'Hermitage/La Saline (**Figure 3**). A total of 44 individuals was haphazardly sampled by hand collecting, during the warm (February) and cold (September) seasons in 2020 ($N_{\text{warm}} = 22$; $N_{\text{cold}} = 22$). A piece of tegument was collected for each individual and preserved in 90% ethanol before DNA extraction.

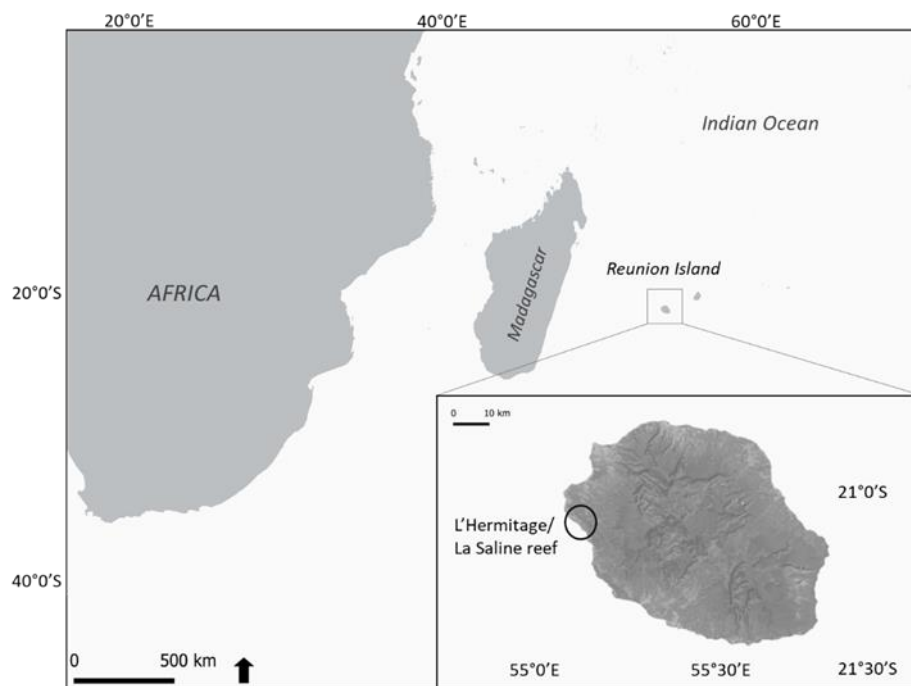


Figure 3: Location of Reunion Island in the southwestern Indian Ocean, and of L'Hermitage/La Saline reef in the west coast of Reunion Island.

Microsatellite library development, primer selection and genotyping

Following manufacturer's protocol, total genomic DNA was extracted using DNeasy Blood & Tissue kit (Qiagen™). Then DNA from eight individuals were equimolarly pooled and shipped to Ecogenics GmbH (Switzerland) for sequencing, following a simple sequence repeat

(SSR) enrichment protocol. Sequences containing SSR motifs were identified, giving 457 promising candidates. Primer pairs were designed for each microsatellite motif, using the pipe 3 of QDD 3.1 (Megléc *et al.*, 2014). Markers with a QDD design note equal to “A” and “B” were retained and checked for possible primer-dimers formation with Multiple Primer Analyzer on the web (ThermoFisher Scientific). Then 96 markers were selected for amplification test depending on the number of repeats (≥ 7), the product size (≥ 95 bp), the absence of primer-dimer and the primer alignment score to the sequence (lowest values were favoured).

The 96 primer pairs selected were then tested for amplification and polymorphism on the eight individuals used previously for library development. Forward primers were indirectly fluorochrome labelled (6-FAM) by adding a universal 19-bp M13 tail at their 5'-end (5'-CAC GAC GTT GTA AAA CGA C-3') (Schuelke, 2000). PCRs were then performed with Veriti™ Thermal Cyclers (Applied Biosystems), in a total volume of 10 μ L with MasterMix Applied 1X (Applied Biosystems), 0.025 μ M of forward primer tagged with the M13 tail, 0.25 μ M of reverse primer, 0.25 μ M of fluorescent dyed M13 tail and ca. 2 $\text{ng}\cdot\mu\text{L}^{-1}$ of genomic DNA. The thermocycling program was the following: 94°C for 5 min + 7 \times (94°C for 30 s, 62°C [-1°C at each cycle] for 30 s, 72°C for 30 s) + 35 \times (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) + 8 \times (94°C for 30 s, 56°C for 30 s, 72°C for 30 s) + 72°C for 5 min. PCR products were genotyped using an ABI3730XL sequencer (Applied Biosystems) at the Plateforme Gentyane (INRAE, Clermont-Ferrand, France). Allelic sizes were determined with GeneMapper 4.0 (Applied Biosystems) using an internal size standard (Genescan LIZ-500, Applied Biosystems). Primer pairs were selected for further development when (1) they amplified in at least five individuals over eight, (2) they were polymorphic (i.e. at least one bi-allelic genotype different from others), (3) they did not amplify multiple fragments, and (4) genotype scoring was deemed reliable.

From that, selected loci were used to genotype the 44 individuals of *H. atra*, following the same protocol as described previously, except that PCR products were genotyped in multiplex (with 6-FAM, VIC, PET and NED dyes; see **Supplementary material 2** for panels).

Data analysis

Clonal propagation

First, the occurrence of potentially identical multi-locus genotypes (MLG; i.e. considering missing data as potentially identical alleles) was investigated among individuals using a custom R 4.0.1 (R Core Team, 2019) script, all individuals sharing the same MLG being member of a same clone. The clonal richness R (Dorken & Eckert, 2001) was calculated for each season, with the formula $R = \frac{(N_{\text{MLG}}-1)}{(N-1)}$, with N_{MLG} , the number of distinct MLGs and N , the number of individuals. Using the same custom R script, based on the distribution of pairwise differences among MLGs, the occurrence of multi-locus clonal lineages (MLL; i.e. MLGs sharing a certain number of alleles, considered close enough to be part of the same lineage) was also investigated. Furthermore, to compare the results obtained with other studies, notably to compare the clonal richness estimated in the sea cucumber *Stichopus chloronotus* at the same site using nine specific microsatellite loci (Pirog *et al.*, 2017), nine out of 21 loci were randomly selected 1,000 times. From these new 1,000 sub-datasets, MLGs were then identified as above (missing data kept and considered as potentially identical alleles, as above) and R was also estimated and compared to previous studies.

Genetic diversity

Further analyses were performed keeping only one representative per MLG. Linkage disequilibrium (LD) was tested on all individuals with Genepop 4.7.0 (Rousset, 2008) and null alleles and other potential technical biases were tested using Micro-Checker 2.2.3 (van Oosterhout *et al.*, 2004). LD tests were corrected using the false discovery rate (FDR) correction for multiple testing. The number of alleles (N_a), the observed and expected heterozygosities (H_o and H_e , respectively) and the inbreeding coefficient (F_{IS}) were estimated with FSTAT 2.9.3.2 (Goudet, 2001). Departures from Hardy-Weinberg equilibrium (HWE) were tested with Genepop 4.7.0 (Rousset, 2008).

Population structure

F_{ST} between both seasons was calculated with the R package “*diveRsity*” (Keenan *et al.*, 2013) to measure the genetic differentiation. Assignment tests were realised with Structure 2.3.4 (five chains with 2×10^6 generation steps after a burn-in of 2×10^5 , assuming admixture and correlated allele frequencies, for K varying from 2 to 12; i.e. the number of MLLs found

plus one; see Results (Pritchard, Stephens & Donnelly, 2000)) and with DAPC using the R package “*adegenet*” 2.0.0 (Jombart, Devillard & Balloux, 2010). Structure and DAPC outputs were summarised and plotted with CLUMPAK (Kopelman *et al.*, 2015). The Evanno’s ΔK (Evanno, Regnaut & Goudet, 2005) and the Bayesian information criterion (BIC) were used to help defining the optimal K .

Cross-amplification testing

Each locus selected for *H. atra* was tested for cross-amplification in four other *Holothuria* species: *H. fuscogilva*, *H. leucospilota*, *H. nobilis* and the flower teatfish *Holothuria* sp. type “Pentard”, this latter having an unclear taxonomic identification. The cross-amplifications were tested on eight individuals of each species (previously collected (Oury, Léopold & Magalon, 2019)) for *H. fuscogilva*, *H. nobilis* and *Holothuria* sp. type “Pentard”, or collected in the reef complex of L’Hermitage/La Saline for *H. leucospilota* (this study), following protocols described above. Meanwhile, the cross-amplification of the microsatellite loci of *H. fuscogilva*, *H. nobilis* and *Holothuria* sp. type “Pentard” (previously developed (Oury *et al.*, 2019)), was also tested on the eight individuals of *H. atra* used for library development.

Results and Discussion

A total of 21 polymorphic microsatellite loci (named Ha01–Ha21; **Table 1**) were selected for *H. atra*, with number of alleles per locus (N_a) ranging from 2 for locus Ha19 to 10 for loci Ha07, Ha10 and Ha21 (**Table 1**).

Table 1: Microsatellite loci developed for *Holothuria atra* over 44 individuals.

For each locus, are indicated the primer sequences (F: forward; R: reverse), the repeat motif, the size range (in base pairs; including the 19 bp M13-tail), and the GenBank accession number. Warm and Cold represent the seasons of sampling; *N* number of individuals that amplified correctly; *Na* number of alleles; *Ho* and *He* observed and expected heterozygosities, respectively; *F_{IS}* inbreeding coefficient (Wright, 1931). Deviation from Hardy-Weinberg equilibrium is indicated: NS non-significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001. Null allele frequencies (*r*) (van Oosterhout *et al.*, 2004) are issued from Micro-Checker 2.2.3 and presence is indicated by values in bold.

Locus	Primer sequence (5'–3')	Motif	Size (bp)	Season	<i>N</i>	<i>Na</i>	<i>Ho</i>	<i>He</i>	<i>F_{IS}</i>	<i>r</i>	GenBank Accession No.
Ha01	F: M13-CCATGTCACATTAGCCAAGC	(AC) ₁₁	184–190	warm	22	4	0.136	0.665	0.795***	0.348	MW999929
	R: GCGATTACAAGCTAAGCGTCA			cold	21	4	0.095	0.612	0.844***	0.366	
Ha02	F: M13-ATGCCCTTTCTTCATGCTCA	(AC) ₁₃	190–212	warm	22	7	0.818	0.845	0.032 ^{NS}	0.006	MW999930
	R: GGCCACTGCAATGAGAAGTC			cold	22	8	0.818	0.838	0.023 ^{NS}	-0.006	
Ha03	F: M13-TGTATGGTGTGCGGCACTT	(AC) ₁₄	94–110	warm	22	6	0.045	0.628	0.928***	0.405	MW999931
	R: CAGCATGTTTCATACGTGGG			cold	22	4	0.091	0.399	0.772***	0.286	
Ha04	F: M13-GGTTAGTGCTAGTCATGAAATGCT	(AC) ₁₆	115–131	warm	22	4	0.409	0.541	0.244 ^{NS}	0.139	MW999932
	R: GAAGCTCCTTTCCACATGA			cold	22	6	0.500	0.731	0.316*	0.156	
Ha05	F: M13-GCCGTGTCGCGTCTCATA	(AC) ₁₇	144–160	warm	22	5	0.364	0.614	0.407***	0.165	MW999933
	R: AGAGGGTAGTTGCACCTTGCT			cold	22	6	0.591	0.711	0.169*	0.04	
Ha06	F: M13-GCAATATCAATGGATGTAAACCAA	(AG) ₁₉	117–133	warm	17	4	0.235	0.691	0.660***	0.292	MW999934
	R: AAAGACAAATTGGAAACTAACACAAA			cold	15	3	0.333	0.529	0.369*	0.144	
Ha07	F: M13-TTATAGGTTCTGGTCTGTCATTGAG	(AT) ₁₃	138–160	warm	22	7	0.864	0.832	-0.038 ^{NS}	-0.034	MW999935
	R: CTACAGCTAGTGACCGGCG			cold	22	10	0.682	0.814	0.162 ^{NS}	0.079	
Ha08	F: M13-AACATTGATTCCGTGTGGGT	(AT) ₁₃	136–158	warm	18	4	0.111	0.657	0.831***	0.369	MW999936
	R: TCTGGGACACTCAGTCTTCTTTC			cold	19	4	0.211	0.656	0.679***	0.301	
Ha09	F: M13-TGGCTGTTGTAAGTCAATTCA	(AT) ₁₅	119–131	warm	21	6	0.333	0.796	0.581***	0.269	MW999937
	R: GGTGGATTATTTAATGCAACGAT			cold	20	6	0.200	0.788	0.746***	0.347	

Ha10	F: M13-AGCAAAGTTAAATTGCTGTAATGC R: GAGATATCACATTAACGACCTAGGAG	(AT) ₁₇	130–176	warm cold	20 22	9 10	0.250 0.500	0.882 0.869	0.716*** 0.425***	0.342 0.201	MW999938
Ha11	F: M13-TGAAGGAGGAGCACTGGACT R: GGTAGGAATTCATTACTGC	(AT) ₁₈	116–140	warm cold	22 21	5 6	0.364 0.333	0.495 0.499	0.265* 0.332 ^{NS}	0.142 0.161	MW999939
Ha12	F: M13-GTGGTCTGGTAGCTGGTTCC R: CGTTCACCAAATTCTTGTGCT	(AAT) ₁₁	212–245	warm cold	22 22	8 5	0.682 0.909	0.738 0.680	0.076*** -0.338 ^{NS}	-0.019 -0.244	MW999940
Ha13	F: M13-CGCCTCCAATCCATTATCT R: TGCTTCTAAACCGCAGTCATT	(AAT) ₃₀	133–160	warm cold	14 18	3 3	0.286 0.389	0.593 0.536	0.519** 0.274**	0.216 0.096	MW999941
Ha14	F: M13-TCAACAGCAACAGTATTAGTAGCG R: GCACTGTAAACCACAAATTTCC	(ACT) ₁₆	125–188	warm cold	22 22	8 9	0.864 0.818	0.863 0.838	-0.001 ^{NS} 0.023 ^{NS}	-0.017 -0.018	MW999942
Ha15	F: M13-TCCAGTCAAGATCCAGCCTT R: GAGCAGAGGTCAATAAATCAGC	(ATC) ₂₃	282–318	warm cold	22 22	6 8	0.636 0.545	0.815 0.789	0.219** 0.309**	0.091 0.145	MW999943
Ha16	F: M13-TGGACTTCAACTGAAACCAGG R: CATAAGAGGGTTGCCGAGTT	(AAAC) ₇	110–118	warm cold	21 22	3 3	0.095 0.045	0.518 0.290	0.816*** 0.843***	0.333 0.276	MW999944
Ha17	F: M13-GGGCACTCTTCGGCGTAT R: TGGAACCAGCTGTTGTAGGTAA	(AAAT) ₇	129–147	warm cold	22 22	4 3	0.045 0.000	0.366 0.589	0.876*** 1.000***	0.310 0.425	MW999945
Ha18	F: M13-CAGTCTACGCTGACTACTGAATGC R: GGTTGGAAACTGGGCTGTTA	(AACT) ₇	108–122	warm cold	21 21	4 5	0.190 0.238	0.523 0.644	0.636* 0.630***	0.284 0.287	MW999946
Ha19	F: M13-AGAGGAATTCAACCCACTGC R: GAAACTGGGCCGTTAAACCT	(AACT) ₈	104–120	warm cold	22 22	2 2	0.773 0.500	0.478 0.381	-0.600 ^{NS} -0.313 ^{NS}	-0.523 -0.293	MW999947
Ha20	F: M13-ATGCAGGGAACAGCTTCATC R: CTGTATTTGTATTGGTGTGACGA	(AATG) ₂₆	219–239	warm cold	18 17	4 6	0.278 0.176	0.559 0.783	0.500** 0.775***	0.206 0.360	MW999948
Ha21	F: M13-CAGGGAGTTGGAAGACTGCT R: ACATCCAACCAGACTTTGGG	(ACAT) ₁₉	176–288	warm cold	22 14	10 6	0.455 0.154	0.825 0.775	0.450** 0.723***	0.227 0.340	MW999949

Clonal propagation

Over the 44 individuals genotyped, no identical multi-locus genotype (MLG) over 21 loci was shared among individuals (clonal richness $R = 1$ for each season). Moreover, the distribution of pairwise differences among MLGs showed one clear antimode at 27 shared alleles (**Supplementary material 3**), meaning that individuals whose MLGs differed from less than 15 alleles (42-27) were considered to belong to the same MLL. Besides a fainter antimode was visible at 38 shared alleles (MLGs differing from less than 4 alleles belong to the same MLL). Considering the greater antimode (27 alleles), the 44 MLGs were regrouped into 11 MLLs (each comprising of 1 to 12 distinct MLGs); considering the smaller antimode (38 alleles), the 44 MLGs were regrouped into 34 MLLs (each comprising of 1 to 4 MLGs). These results indicate that asexual reproduction could occur in the population of *H. atra* from Reunion Island as already highlighted by several studies between 1993 and 2000 with fission rate varying from 14.9% to 19.6% (Conand, 1995, 2004). Moreover, although some individuals did not show signs of fission when monitored, they could be issued from a previous asexual reproduction, potentially underestimating this percentage. Therefore, thanks to these results on the number of individuals issued from asexual reproduction, we could expect to find some clones in *H. atra* population. The absence of identical MLGs in our dataset may be due to the high number of loci used decreasing mathematically the probability to find two identical MLGs over the 42 alleles, and also their inherent high polymorphism. Indeed, reducing the number of loci used, to be able to compare clonal richnesses among studies (1,000 sub-datasets from the random selection of nine microsatellites over the 21 specific for *H. atra*), the mean clonal richness in *H. atra* reached 0.91 ± 0.002 [\pm SE; min: 0.63 (28 MLGs); max: 1 (44 MLGs)] and remained extremely high comparatively to the one found in *S. chloronotus* populations ($R = 0.09$) (Pirog *et al.*, 2017). Indeed, in *S. chloronotus*, each population (based on 64 sampled individuals per site \times season) was comprised of one to three dominant MLGs (Pirog *et al.*, 2017), although the percentage of *S. chloronotus* individuals issued from asexual reproduction estimated based on body shape few decades ago was quite low (6.5%) (Conand *et al.*, 1998).

Thus, the number of individuals issued from fission is not a good predictor for clonal richness. Indeed, in *H. atra*, more individuals than in *S. chloronotus* participate to clonal

propagation, but these individuals are more genetically different between each other, while in *S. chloronotus*, fewer individuals participate to asexual reproduction, but as they are members of the same clone, the clonal richness is much smaller (tenfold). As a conclusion, clonal propagation in *H. atra* does occur, but in low proportion in the studied population.

Genetic diversity

Presence of null alleles was detected in 14 loci, at frequencies ranging from 0.156 to 0.425 (**Table 1**). Significant linkage disequilibrium was found in 273 of the 420 tests carried between all pairs of loci within each season separately. The observed heterozygosity (H_o) ranged from 0.000 for locus Ha17 to 0.909 for locus Ha12 while the expected heterozygosity (H_e) ranged from 0.290 for locus Ha16 to 0.882 for locus Ha10. Four loci (Ha02, Ha07, Ha14 and Ha19) were at Hardy-Weinberg equilibrium (HWE) for both seasons, and three others (Ha04, Ha11 and Ha12) were at HWE for one season or the other. The remaining loci showed significant heterozygote deficiency (F_{IS} ranging from 0.169* for Ha05 in cold season to 1.000*** for Ha17 in cold season; **Table 1**). The heterozygote deficiencies found are potentially due to the presence of null alleles. Indeed, deviation from HWE due to null alleles appears frequent in sea cucumbers (Oury *et al.*, 2019; Pierrat *et al.*, 2020). Our results on the low asexual propagation (no repeated MLG and few MLLs) fit well with the deficit of heterozygote observed, as a high level of asexual reproduction and so a high level of clonal individuals in *H. atra* populations would lead to excesses of heterozygotes (Balloux, Lehmann & de Meeûs, 2003).

Population structure

From $K = 2$ to $K = 8$, Structure and DAPC assignments were not congruent (Online resource 4). However, starting from $K = 9$, both methods gave similar results, but did not suggest any structuring between both seasons (**Supplementary material 4**). The optimal K was 7 according to the Evanno's ΔK , whereas it was 11 according to the BIC (**Supplementary material 4**). Meanwhile, at $K = 11$, the partitioning corresponded to the 11 MLLs found for the widest antimode (27 alleles; **Figure 4**), confirming the existence of these MLLs. The global F_{ST} between both seasons (0.019^{NS}) suggests no genetic differentiation and thus, temporal

stability in this *H. atra* population. The stability observed through time for *H. atra* was also found for *S. chloronotus* (Pirog *et al.*, 2017).

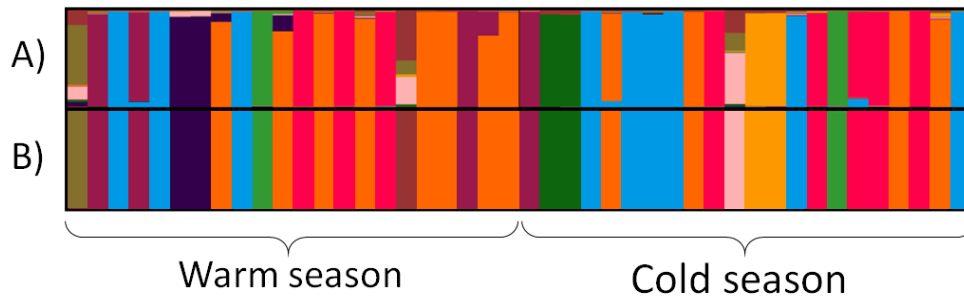


Figure 4: Results of the assignment tests for *Holothuria atra* individuals from both seasons. A) Structure plot at $K = 11$ and B) DAPC assignments at $K = 11$.

Cross-amplification

Cross-amplification tests revealed that, among the 21 microsatellite loci of *H. atra* isolated in this study, 17 cross-amplified in at least one other species (**Table 2**). Almost all the loci amplified individuals from *H. leucospilota* and were polymorphic (except Ha09), but with a low number of individuals correctly amplified (**Table 2**). However, no locus cross-amplified in *H. nobilis* and *Holothuria* sp. type “Pentard”, and only one (Ha07) amplified three individuals from *H. fuscogilva* (**Table 2**). Finally, among the 60 *H. fuscogilva*, *H. nobilis* or *Holothuria* sp. type “Pentard” loci developed in (Oury *et al.*, 2019), none amplified in *H. atra*. Indeed, *H. fuscogilva*, *H. nobilis* and *Holothuria* sp. type “Pentard” are genetically distant from *H. atra*, probably more than *H. leucospilota* (Skillings *et al.*, 2011).

In conclusion, the isolation and characterization of these 21 loci for *H. atra* highlighted (1) the low occurrence of asexual propagation for the population of *H. atra* from Reunion Island and (2) that the genetic structure and diversity of this population seem stable through time. Genetic investigations including more individuals will allow to confirm these preliminary findings. Besides, these loci will be very useful in studying the genetic diversity of *H. atra* populations from other localities and will help to identify more precisely its conservation status for efficient management. Up to now, genetic information globally increases for harvested sea cucumber species but remains insufficient for conservation purposes.

Table 2: Cross-amplification for the 21 microsatellite loci of *Holothuria atra* on four other *Holothuria* species: *H. fuscogilva*, *H. leucospilota*, *H. nobilis* and *Holothuria* sp. type “Pentard”.

For each locus and each taxon, are indicated *N*, the number of tested individuals that amplified correctly over eight; *Na*, the number of alleles and the specific size range (in base pairs, 19-bp M13-tail included)

Locus	<i>H. fuscogilva</i>			<i>H. leucospilota</i>			<i>H. nobilis</i>			<i>Holothuria</i> sp. type “Pentard”		
	<i>N</i>	<i>Na</i>	Size (bp)	<i>N</i>	<i>Na</i>	Size (bp)	<i>N</i>	<i>Na</i>	Size (bp)	<i>N</i>	<i>Na</i>	Size (bp)
Ha01	0	–	–	0	–	–	0	–	–	0	–	–
Ha02	0	–	–	8	5	206–248	0	–	–	0	–	–
Ha03	0	–	–	4	2	118–124	0	–	–	0	–	–
Ha04	0	–	–	5	3	123–129	0	–	–	0	–	–
Ha05	0	–	–	3	3	226–258	0	–	–	0	–	–
Ha06	0	–	–	0	–	–	0	–	–	0	–	–
Ha07	4	3	123–129	0	–	–	0	–	–	0	–	–
Ha08	0	–	–	6	4	107–117	0	–	–	0	–	–
Ha09	0	–	–	7	1	113	0	–	–	0	–	–
Ha10	0	–	–	3	3	110–114	0	–	–	0	–	–
Ha11	0	–	–	8	5	100–128	0	–	–	0	–	–
Ha12	0	–	–	8	4	119–127	0	–	–	0	–	–
Ha13	0	–	–	7	5	126–156	0	–	–	0	–	–
Ha14	0	–	–	0	–	–	0	–	–	0	–	–
Ha15	0	–	–	2	2	302–435	0	–	–	0	–	–
Ha16	0	–	–	4	4	151–199	0	–	–	0	–	–
Ha17	0	–	–	8	4	141–211	0	–	–	0	–	–
Ha18	0	–	–	7	3	151–195	0	–	–	0	–	–
Ha19	0	–	–	2	2	131–151	0	–	–	0	–	–
Ha20	0	–	–	0	–	–	0	–	–	0	–	–
Ha21	0	–	–	8	3	209–335	0	–	–	0	–	–

Statements & Declarations

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Competing interests

The authors declare no conflict of interest.

Authors' contributions

JP, NL and NO collected samples and did laboratory steps. JP, HM and NO analysed the data. JP wrote the original draft. JP, HM and NO reviewed and edited the manuscript.

Ethics approval

Only a piece of tegument (non-lethal) for each specimen was collected (DEAL decision N°2020-37).

Availability of data and material

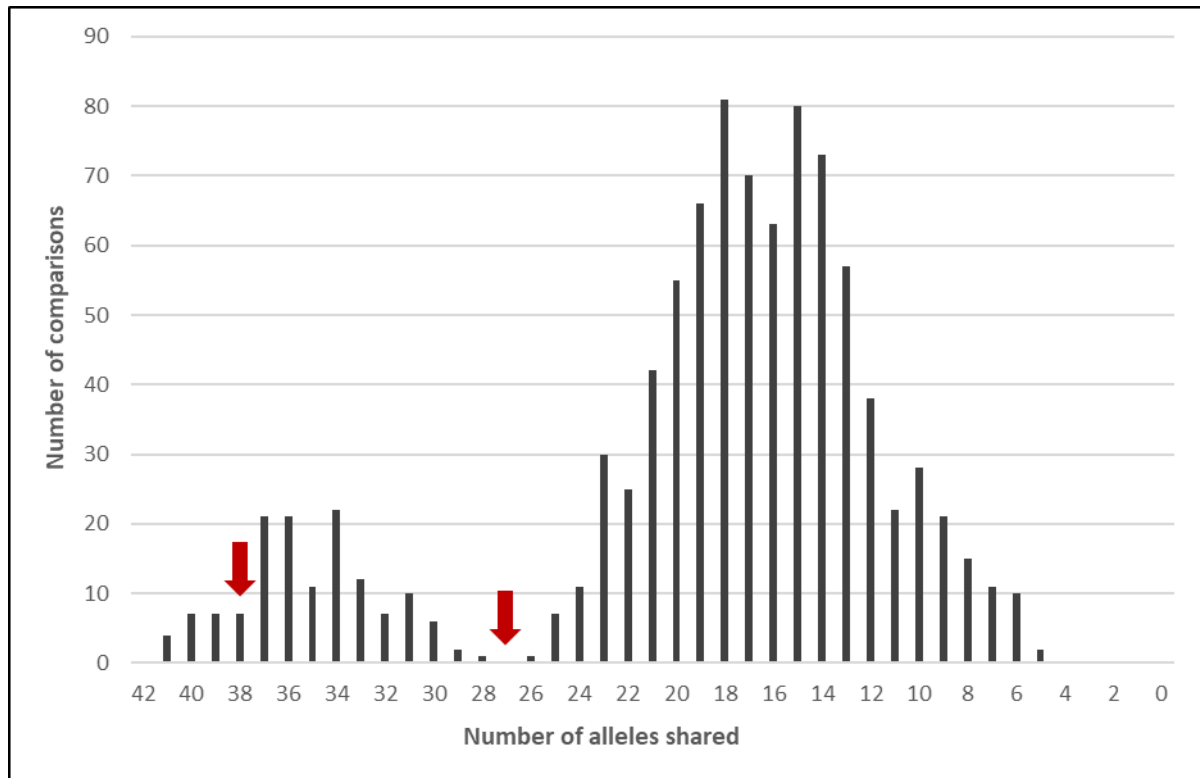
Accession Numbers: Primer sequences are deposited on GenBank with accession numbers from MW999929 to MW999949.

Supplementary material

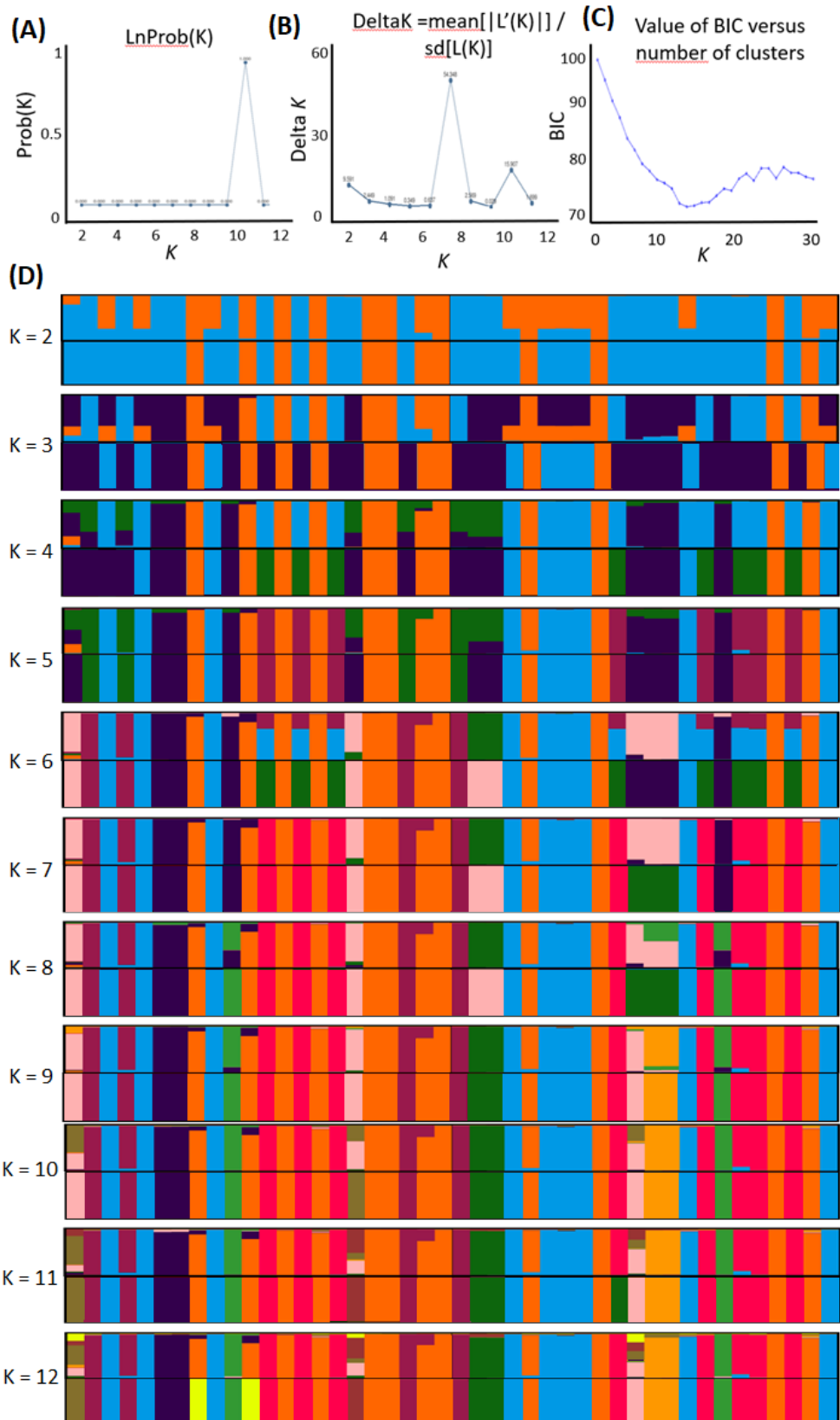
Supplementary material 2: Panels for multiplexing the 21 *Holothuria atra* microsatellite loci.

Panel	Locus	Motif	Dye	Size range (bp)
1	Ha19	(AACT) ₈	6-FAM	104–120
	Ha09	(AT) ₁₅	PET	119–131
	Ha13	(AAT) ₃₀	NED	133–160
	Ha08	(AT) ₁₃	VIC	136–158
	Ha21	(ACAT) ₁₉	6-FAM	176–288
2	Ha03	(AC) ₁₄	6-FAM	94–110
	Ha06	(AG) ₁₉	NED	117–133
	Ha05	(AC) ₁₇	VIC	144–160
	Ha01	(AC) ₁₁	6-FAM	184–190
3	Ha18	(AACT) ₇	6-FAM	108–122
	Ha11	(AT) ₁₈	NED	116–140
	Ha07	(AT) ₁₃	VIC	138–160
	Ha02	(AC) ₁₃	6-FAM	190–212
4	Ha16	(AAAC) ₇	6-FAM	110–118
	Ha04	(AC) ₁₆	NED	115–131
	Ha10	(AT) ₁₇	VIC	130–176
	Ha20	(AATG) ₂₆	6-FAM	219–239
5	Ha17	(AAAT) ₇	6-FAM	129–147
	Ha14	(ACT) ₁₆	NED	125–188
	Ha12	(AAT) ₁₁	VIC	212–245
	Ha15	(ATC) ₂₃	6-FAM	282–318

Supplementary material 3: Distribution of pairwise differences (number of alleles shared) among multi-locus genotypes (MLG). Red arrows show potential antimodes separating MLGs of the same multi-locus lineage (MLL) from those of different MLLs.



Supplementary material 4: Results of the assignment tests for *Holothuria atra*. (A) mean likelihood over the five iterations of the same K, (B) Evanno's ΔK distribution, (C) BIC distribution and (D) plots from K = 2 to K = 12 for Structure (above) and DAPC (below).



Sex or fission? Genetics highlight differences in reproductive strategies of two sympatric fissiparous sea cucumber species in Reunion Island (southwestern Indian Ocean)

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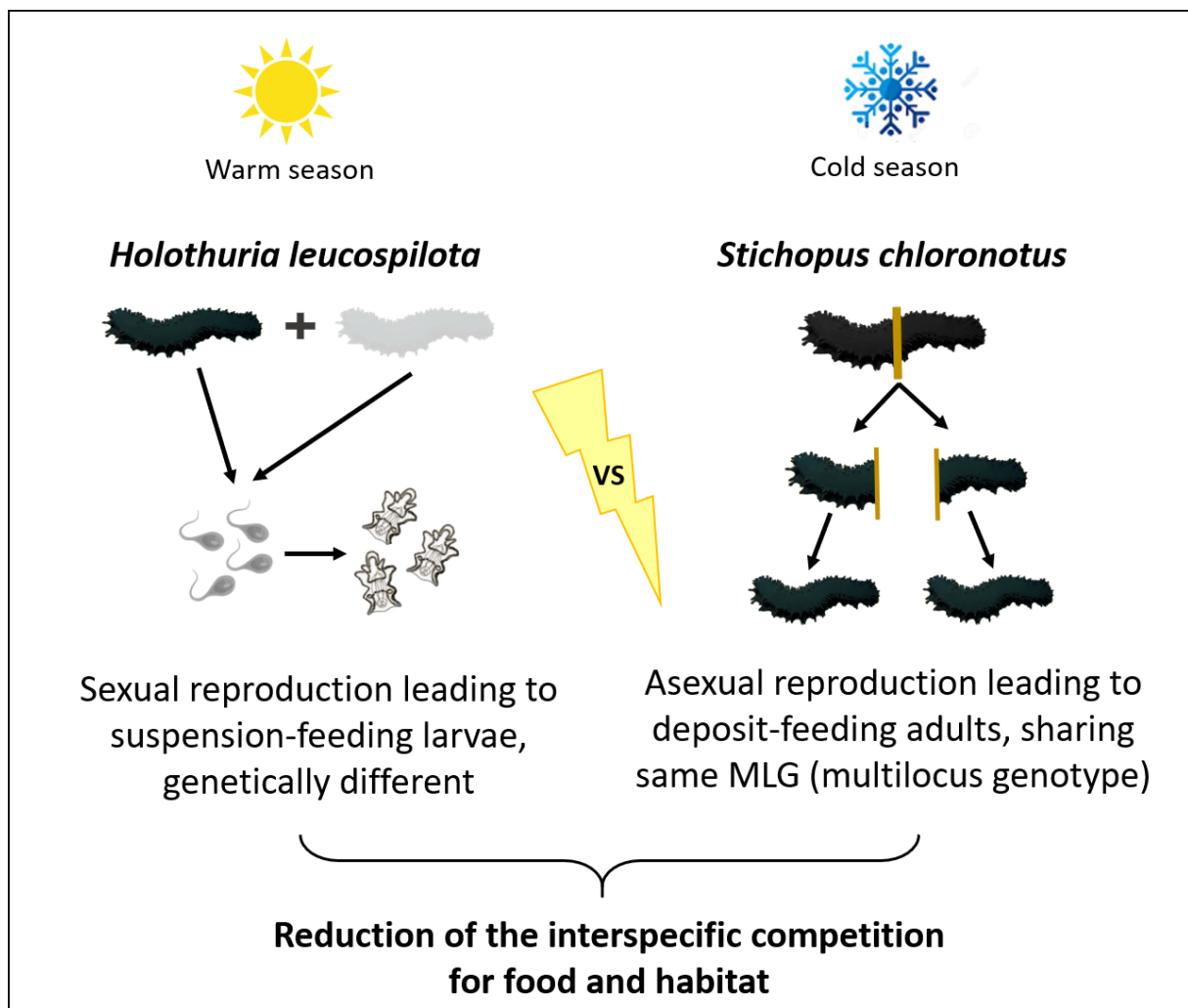
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Abstract

Holothuria leucospilota and *Stichopus chloronotus* are among the most widespread tropical sea cucumber species usually harvested for food and medicine in Asian countries, for which natural stocks have collapsed worldwide. Both species can reproduce sexually and asexually, and a better understanding of their reproductive strategy can provide useful information for conservation purposes. To describe the genetic structure and diversity of sympatric populations from these species in space and time, individuals were sampled over different sites and seasons in Reunion Island (Southwestern Indian Ocean). They were genotyped using 24 and 9 specific microsatellite markers for *H. leucospilota* and *S. chloronotus*, respectively. Multi-locus genotypes (MLG) and lineages (MLL) were identified, and analyses of population structure were performed among sites and seasons. No repeated MLG nor MLL were found for *H. leucospilota*, demonstrating the absence of asexual reproduction. Populations of *H. leucospilota* were not genetically differentiated, acting as a metapopulation, with larval

exchanges within the reef. Contrarily, repeated MLGs were found for *S. chloronotus* and all populations were genetically differentiated. Asexual reproduction seems to reach a high level for this species (mean clonal richness = 0.24). For both species, genetic structure was stable through seasons. Thus, these sympatric fissiparous sea cucumber species use two different strategies of reproduction, which may allow them to reduce interspecific competition.

Graphical Abstract



Keywords

Holothuria leucospilota • *Stichopus chloronotus* • Sea cucumber • Microsatellite • Genetic structure • Clonal propagation • Reproductive strategies • Sympatric species • Interspecific competition

Introduction

Sea cucumbers are among the most abundant benthic megafauna species in many ecosystems, such as deep-sea (Beliaev, 1989; Kuhnz *et al.*, 2014), corals reefs (Wolfe & Davey, 2020) and shallow marine habitats (Conde, Diaz & Sambrani, 1991). Among the 1750 species currently described (WoRMS, 2021), approximately 70 species are harvested (Purcell *et al.*, 2016) for food (commonly known as “*bêche-de-mer*” or “*tre pang*”), traditional medicine and aphrodisiacs for many Asian countries (Conand, 1990, 2018; Toral-Granda *et al.*, 2008). Only two species present a complete process of domestication for large-scale aquaculture purposes, from egg spawning to brood- stock maintaining: *Holothuria scabra* (Hamel *et al.*, 2022) and *Apostichopus japonicus* (Yang, Hamel & Mercier, 2015a). During the last decades, fisheries of sea cucumbers have quadrupled (Rahman & Yusoff, 2017) and their coastal populations have been decimated by hand collecting (Friedman *et al.*, 2011) to satisfy the increasing demand of the Asian market (Rahman, Yusoff & Arshad, 2015). The depletion of stocks of high-commercial value species has led to a shift toward low-commercial value species (Purcell, Samyn & Conand, 2012b). Fishing regulations and management plans are insufficient to restore some local populations (Friedman *et al.*, 2011). Therefore, data on demographical parameters and genetic structure of sea cucumber populations are needed to establish efficient management plans, to increase the number of species for aquaculture and, consequently, to avoid the depletion of natural stocks and the loss of ecosystem services provided by sea cucumbers.

Holothuria (Mertensiothuria) leucospilota (Brandt, 1835) and *Stichopus chloronotus* (Brandt, 1835) are on the original FAO list of commercial species (Purcell *et al.*, 2012b). *Holothuria leucospilota*, commonly called “black long sea cucumber” or “white thread fish”, is one of the most widespread sea cucumber species, inhabiting the Western Central Pacific, Asia and Indian Ocean and living on soft substrates in coral reefs and shallow coastal habitats (Purcell *et al.*, 2012b). *Stichopus chloronotus*, called “greenfish”, is also largely distributed throughout the Indo-West Pacific, living on coarse corals and coral rubbles (Purcell *et al.*, 2012b). Dried body wall of *H. leucospilota*, considered as low-commercial value species, can be sold up to 5 USD.kg⁻¹ in the Philippines (Purcell *et al.*, 2012b), whereas up to 95 USD.kg⁻¹

for *S. chloronotus*, considered as medium-commercial value species (Purcell, 2014). Individuals are harvested by hand collecting at low tide, mainly in Madagascar and many Asian countries, where low and medium-value species are fished without any restriction. Moreover *S. chloronotus* is exploited in artisanal and semi-industrial fish-eries, mostly in Mauritius (Purcell *et al.*, 2012b). Gonads of *H. leucospilota* are traditional subsistence in Cook Island culture (Drumm & Loneragan, 2005) and active substances have been isolated from the body wall for medicine applications, such as antibacterial and antifungal (Adibpour *et al.*, 2014), antioxidant (Gozari *et al.*, 2018; Yuan *et al.*, 2019) and antitumoral (Zhang *et al.*, 2009) activities. *Stichopus chloronotus* is harvested for subsistence consumption in some islands and is commercially important for food in many Asian countries (Purcell *et al.*, 2012b). Increasing knowledge on the genetic structure and diversity of these two species would allow to better understand their ecology and to preserve natural stocks from depletion by helping their domestication for aquaculture purposes.

In contrast with some certain localities where they are highly harvested, *H. leucospilota* and *S. chloronotus* are distinguished by their exceptional densities in Reunion Island (Southwestern Indian Ocean), which homes 38 species of sea cucumber (Conand, Trentin & Mulochau, 2018). Populations of *H. leucospilota* and *S. chloronotus* are found in sympatry in the west and south coasts of Reunion Island, mainly in the fringing reef of L'Hermitage/La Saline and Étang-Salé. They are monitored since 25 years and the observed densities ranged between 0.15 and 3.7 ind.m⁻² depending on the location (Conand, Morel & Mussard, 1997; Conand *et al.*, 1998; Conand & Mangion, 2002; Cuvillier, 2016). These species are among the 16 species of sea cucumbers having the ability to reproduce both sexually through gamete spawning and asexually by transversal fission (Dolmatov, 2014). Sexual reproduction leads to the first larval stage (auricularia), which feeds on phytoplankton whereas, in asexual reproduction, one individual undergoes fission, leading to two deposit-feeder adults. The fission rates for populations of *H. leucospilota*, estimated thanks to a visual census of individuals undergoing fission, ranges between 5% in Reunion Island (Conand *et al.*, 1997) and 28% in Australia (Purwati, 2004). Although *H. leucospilota* is one of the most common sea cucumber species, only two studies have investigated its genetic diversity (Dai *et al.*, 2015; Shangguan *et al.*, 2015), and no study has ever evaluated the genetic structure and clonal

propagation of its populations using genetic tools. The number of individuals of *S. chloronotus* performing fission has been estimated to 17% at Reunion Island (Conand *et al.*, 1998), using the same method as for *H. leucospilota* (Conand *et al.*, 1997). However, two decades later, the clonal richness was analysed using nine microsatellite markers (Pirog *et al.*, 2017) and authors concluded that it was extremely low ($R = 0.09$), meaning that many individuals of *S. chloronotus* are clones, and therefore, have participated or participate to asexual reproduction. Visual census is not a good predictor to estimate clonal propagation, as concluded by a study on *Holothuria atra* (Pierrat *et al.*, 2022c). Genetic analyses thus need to be realised on *H. leucospilota* to evaluate the importance of asexual reproduction in the populations of Reunion Island.

Here, we focused on the populations of *H. leucospilota* and *S. chloronotus* from Reunion Island, collected at different sites and dates, to (1) identify clones to estimate the level of asexual propagation, (2) estimate the genetic diversity of these populations and (3) estimate the genetic structure among populations of each species, to investigate a potential genetic connectivity among reefs and seasons, and the impact of the two reproductive strategies through time.

Materials and Methods

Sampling design

Sampling was carried out on the west coast of Reunion Island (southwestern Indian Ocean; 21°06'S, 55°31'E), in the fringing reefs of L'Hermitage/La Saline and Étang-Salé (**Figure 5**). Individuals of *H. leucospilota* and *S. chloronotus* were haphazardly sampled by hand collecting in the back-reef depression and stored at -80°C before analyses.

Spatial sampling

Individuals of *H. leucospilota* were sampled only along the reef of L'Hermitage/La Saline because none was found in the reef of Étang-Salé. Three sites with a high density ($> 1 \text{ ind.m}^{-2}$, (Conand & Mangion, 2002)) were chosen: MNS (Maître-Nageur-Sauveteur, $2.6 \pm 0.2 \text{ ind.m}^{-2}$), PLA (Planch'Alizé, $1.0 \pm 0.1 \text{ ind.m}^{-2}$) and TE (Trou d'Eau, $1.2 \pm 0.1 \text{ ind.m}^{-2}$)

(**Figure 5**). Two additional sites with low densities ($< 1 \text{ ind.m}^{-2}$, personal observations): were studied: CAP (Cap Méchant) and PTE (Petit Trou d’Eau) (Figure 1). Individuals of *S. chloronotus* were sampled in the same high density sites as in a previous study (Pirog *et al.*, 2017): PAS (Passe de l’Hermitage, corresponding to HIGH1 in (Pirog *et al.*, 2017)); with a density of $0.8 \pm 0.1 \text{ ind.m}^{-2}$, TE (Trou d’Eau; HIGH2; $1.2 \pm 0.1 \text{ ind.m}^{-2}$), both in the reef of L’Hermitage/La Saline, and ES (Étang-Salé; HIGH3; $0.7 \pm 0.1 \text{ ind.m}^{-2}$) in the reef of Étang-Salé.

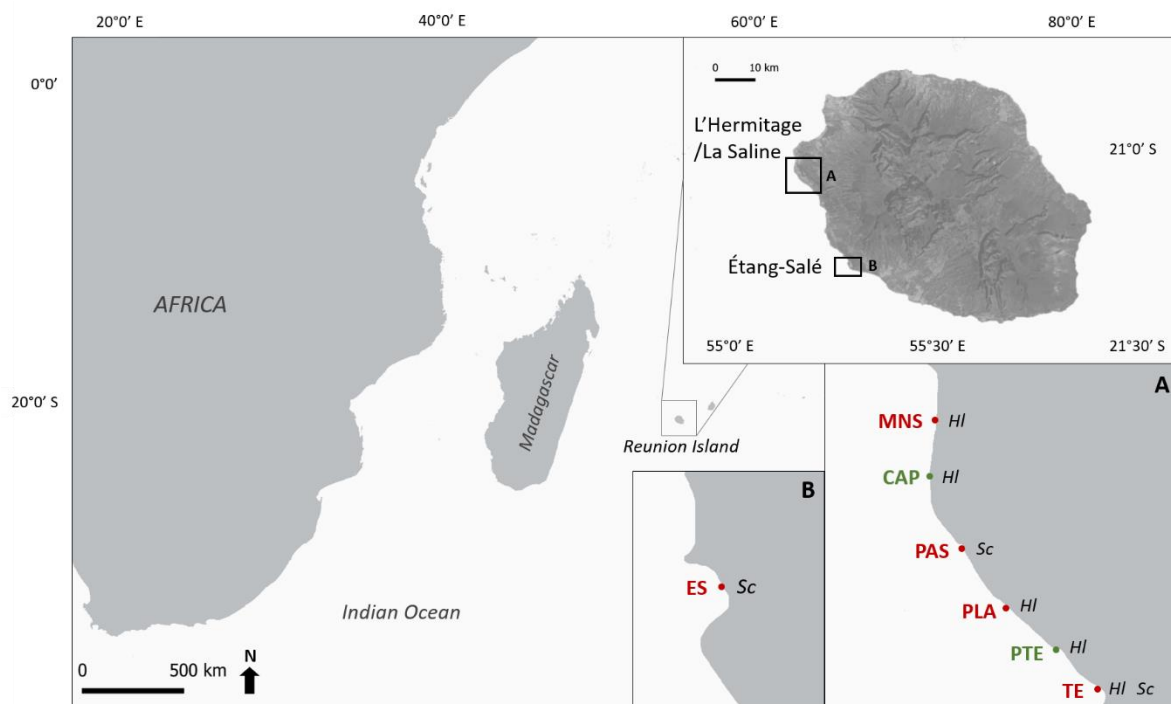


Figure 5: Location of the sites of sampling in each reef.

High density sites in red: MNS: Maître-Nageur-Sauveteur, PAS: Passe de l’Hermitage, PLA: Planch’Alizé, TE: Trou d’Eau, ES: Étang-Salé

Low density sites in green: CAP: Cap-Méchant, PTE: Petit Trou d’Eau

HI: *Holothuria leucopilota* and Sc: *Stichopus chloronotus* indicate where individuals were harvested

Temporal sampling of both species

To analyse the effect of the two strategies of reproduction (sexual and asexual) on the genetic structure, sampling was performed for three consecutive seasons: the cold season 2019 (S1_{cold}: austral winter in September 2019), warm season 2020 (S2_{warm}: austral summer in February 2020) and cold season 2020 (S3_{cold}: austral winter in September 2020). For each site,

24 individuals were sampled, except for low-density sites (CAP and PTE), where only 12 individuals were collected due to the low densities observed. Sampling design for both species is summarised in **Supplementary material 5**. For a given species, a population is considered as all the individuals sampled at a given site and a given season.

Laboratory steps

Total genomic DNA was extracted from a small piece of tegument, using the DNeasy Blood & Tissue kit (Qiagen™, Hilden, Germany), following the manufacturer's protocol. Individuals of *H. leucospilota* and *S. chloronotus* were genotyped using 24 (Pierrat *et al.*, 2022b) and nine (Pirog *et al.*, 2017) specific microsatellite loci, respectively. Forward primers were indirectly fluorochrome labelled (6-FAM, VIC, NED) and were multiplexed post-PCR in panels (**Supplementary material 2** and **Table 3**, for *H. leucospilota* and *S. chloronotus* respectively). PCRs were then performed with Veriti™ Thermal Cyclers, in a total volume of 10 µL with MasterMix Applied 1X (Applied Biosystems, Waltham, MA, USA), 0.025 µM of forward primer tagged with the M13 tail, 0.25 µM of reverse primer, 0.25 µM of fluorescent dyed M13 tail and ca. 2 ng.µL⁻¹ of genomic DNA. The thermocycling program was the following : 94 °C for 5 min and 7 x (94 °C for 30 s, 62 °C [-1 °C at each cycle] for 30 s, 72 °C for 30 s) and 35 x (94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and 8 x (94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s) and 72 °C for 5 min. PCR products were genotyped using an ABI3730XL sequencer (Applied Biosystems) at the Plateforme Gentyane (INRAE, Clermont-Ferrand, France). Allelic sizes were determined with GeneMapper 4.0 (Applied Biosystems) using an internal size standard (Genescan LIZ-500, Applied Biosystems).

Table 3: Panels for multiplexing the 9 *Stichopus chloronotus* microsatellite loci

Panel	Locus	Primer sequence (5'-3')	Dye	Specific size range (bp)
1	Sc10	F: CGCCTCTAATCTCAAATTGTCG R: TGCGGTCTTCCTTGTCTC	6-FAM	142–164
	Sc09	F: CCAATGCTTTGATTCCAGG R: CCAACTTGACATATTGAG	VIC	200–206
	Sc43	F: CGTGACATACTTCTAGC R: GAGATCACTTAGAGTTACGC	6-FAM	233–239
	Sc01	F: CGGGAAGCATTAAGTGC R: GCGATACGGATCCTTGTGG	VIC	323–326
2	Sc24	F: CGTGGTTAAATTCCTAGGTATAGAG R: CTGGAATAAACCTGATGTAC	6-FAM	148–158
	Sm007	F: CACCGCTTTGAATTTGTAG R: ACTGTAGGCAATGAATGA	VIC	172–176
	Sc29	F: GTAGCCATAAATCATTG R: GACCAACCCACACAGCAAG	NED	212–218
	Sc33	F: CTGGTTCGGATTCACATAG R: CTAATTACGGTGAACTTCC	6-FAM	260–266
	Sm014	F: CACGGACAGTGGTCACAAG R: TGAGATAGAGCGTTTACGAG	VIC	355–365

Data analyses

Clonal identification and propagation

For each species, the occurrence of identical multi-locus genotypes (MLG) was investigated (considering missing data as potentially identical alleles for *H leucospilota*), with a custom R (R Core Team, 2019) script. Then, clonal richness R (Dorken & Eckert, 2001) was calculated for each population, with the formula $R = \frac{(N_{MLG}-1)}{(N-1)}$, with N_{MLG} , the number of distinct MLGs and N , the number of individuals. Finally, using the same custom R script, the occurrence of multi-locus clonal lineages (MLL; i.e., MLGs sharing a certain number of alleles, considered close enough to be part of the same lineage) was also investigated based on the distribution of pairwise differences among MLGs. If MLLs are present in the population, the distribution of pairwise differences must show a clear antimode in the number of alleles shared, corresponding to the threshold from which all MLGs with less allelic differences belong to the same MLL.

Meanwhile, for *H. leucospilota* populations, to be able to compare the numbers of MLGs and the subsequent clonal richnesses with those of *S. chloronotus* (Pirog *et al.*, 2017) and this study) and *H. atra* (Pierrat *et al.*, 2022c), 1000 sub-datasets were created by randomly sampling 1000 times 9 out of the 24 loci used. MLGs and clonal richnesses were then calculated for each sub-dataset, considering missing data as potentially identical alleles, thanks to a custom R (R Core Team, 2019) script.

Genetic diversity

The number of alleles (N_a), the number of private alleles (N_p), the observed and expected heterozygosities (H_o and H_e , respectively) and the inbreeding coefficient (F_{IS}) (Wright, 1931) were estimated with FSTAT 2.9.3.2 (Goudet, 2001) for each population *H. leucospilota* and *S. chloronotus*, keeping all individuals as reported in (Pirog *et al.*, 2017) for comparison purposes. Departures from the Hardy–Weinberg equilibrium (HWE) were tested with Genepop 4.7.0 (Raymond & Rousset, 1995; Rousset, 2008).

Population structure and differentiation

Bayesian clustering analyses were realised with Structure 2.3.4 (Pritchard *et al.*, 2000) for both species, keeping only one representative per MLG for each population. Five chains with 2×10^6 generation steps after a burn-in of 2×10^5 were run, assuming admixture and correlated allele frequencies, for K varying from 2 to 5. Discriminant Analysis of Principal Components (DAPC) was also performed using the R package adegenet 2.0.0 (Jombart *et al.*, 2010). Structure and DAPC outputs were summarised and plotted with CLUMPAK (Kopelman *et al.*, 2015). To find the optimal K from the Structure outputs, we used the ΔK statistic (Evanno *et al.*, 2005) in CLUMPAK (Kopelman *et al.*, 2015). The Bayesian Information Criterion (BIC) from the DAPC output was estimated in R. For *S. chloronotus*, Structure and DAPC analyses were also realised keeping only one representative per MLG for each site, with all seasons combined.

F_{ST} (Weir & Cockerham, 1984) were calculated between each pair of populations keeping all individuals for both *H. leucospilota* and *S. chloronotus*, and 1000 bootstraps were

realised to test whether F_{ST} values were significantly different from zero using Arlequin 3.5.2 (Excoffier & Lischer, 2010) and the False Discovery Rate for multiple tests.

Results

MLG and clone identification

*Clonal diversity of *Holothuria leucospilota**

On the 288 individuals genotyped for *H. leucospilota*, 47 did not amplify with at least 10 markers (42 from S1_{cold}, 2 from S2_{warm} and 3 from S3_{cold}), thus, we decided to remove them from the rest of the analyses. Only 74 individuals (25%) presented MLGs without missing data over the 241 remaining individuals, but none of these MLGs were shared among individuals (clonal richness $R = 1$). The analysis keeping missing data as potential identical alleles (i.e., over-estimating the presence of clones) showed that all individuals have their own MLG (**Table 4**), therefore, no shared clone was present in the populations of *H. leucospilota*. Clonal richness reached 1 whichever the site density (low or high) or the season (cold or warm) (**Table 4**). Moreover, no clear antimode was found on the distribution of pairwise differences among MLGs (**Supplementary material 6**), meaning that each MLG is too distant from the others and constitutes a distinct MLL on its own.

The absence of repeated MLG and MLL in the populations of *H. leucospilota* may be due to the high number of microsatellite markers used (i.e., 24 markers), decreasing the probability to find two identical MLGs over the 48 alleles identified. Random selection (1000 sub-datasets) of nine microsatellite markers, over the 24 used for genotyping individuals, revealed that the mean clonal richness reached $0.99 (\pm 5.8 \times 10^{-6})$ (\pm SE; min: 0.996; max: 1) and that the mean number of MLGs identified was 240.99 (over 241 individuals; min: 240; max: 241), confirming the absence of repeated MLG in the populations of *H. leucospilota* from Reunion Island.

Table 4: Indices of genetic diversity for *Holothuria leucospilota* populations from Reunion Island

Season	Site	%NA	N	N_{MLG}	R	N_a	N_p	H_o	H_e	F_{IS}
S1	MNS	58.33	10	10	1	6.17 ± 0.58	0.96 ± 0.24	0.39 ± 0.05	0.82 ± 0.03	0.53*** ± 0.05
	CAP	50.00	6	6	1	4.96 ± 0.39	0.50 ± 0.17	0.41 ± 0.06	0.82 ± 0.03	0.48*** ± 0.07
	PLA	37.50	15	15	1	7.04 ± 0.57	1.08 ± 0.21	0.41 ± 0.04	0.80 ± 0.03	0.50*** ± 0.04
	PTE	41.67	7	7	1	5.58 ± 0.49	0.46 ± 0.16	0.45 ± 0.06	0.80 ± 0.04	0.43*** ± 0.07
	TE	33.33	16	16	1	7.38 ± 0.65	0.92 ± 0.18	0.33 ± 0.04	0.80 ± 0.03	0.58*** ± 0.04
S2	MNS	4.17	23	23	1	10.38 ± 0.80	0.96 ± 0.20	0.57 ± 0.04	0.82 ± 0.02	0.31*** ± 0.04
	CAP	0.00	12	12	1	7.67 ± 0.64	0.42 ± 0.18	0.54 ± 0.05	0.79 ± 0.03	0.31*** ± 0.05
	PLA	0.00	24	24	1	10.46 ± 0.82	0.75 ± 0.24	0.52 ± 0.04	0.82 ± 0.02	0.37*** ± 0.04
	PTE	0.00	12	12	1	8.08 ± 0.54	0.50 ± 0.16	0.55 ± 0.05	0.83 ± 0.02	0.34*** ± 0.05
	TE	4.17	23	23	1	10.17 ± 0.87	0.63 ± 0.19	0.53 ± 0.04	0.80 ± 0.003	0.34*** ± 0.04
S3	MNS	0.00	24	24	1	10.46 ± 0.70	1.13 ± 0.23	0.53 ± 0.05	0.83 ± 0.02	0.37*** ± 0.05
	CAP	0.00	12	12	1	7.67 ± 0.49	0.33 ± 0.10	0.50 ± 0.05	0.82 ± 0.02	0.38*** ± 0.06
	PLA	12.50	21	21	1	9.71 ± 0.62	0.58 ± 0.15	0.59 ± 0.04	0.81 ± 0.02	0.27*** ± 0.04
	PTE	0.00	12	12	1	7.58 ± 0.58	0.29 ± 0.11	0.52 ± 0.05	0.81 ± 0.03	0.35*** ± 0.05
	TE	0.00	24	24	1	10.54 ± 0.72	1.38 ± 0.26	0.53 ± 0.04	0.83 ± 0.03	0.36*** ± 0.04

%NA: percentage of missing data; N: number of individuals that amplified for at least with 10 markers; N_{MLG} : number of distinct multi-locus genotypes; R: clonal richness; N_a : mean number of alleles; N_p : mean number of private alleles; H_o and H_e : observed and expected heterozygosities, respectively; F_{IS} : inbreeding coefficient and significant deviation from Hardy-Weinberg Equilibrium (***: $P < 0.001$). Standard errors are indicated following mean values. Grey lines represent low density sites.

High density sites: MNS: Maître-Nageur-Sauveteur, PLA: Planch'Alizé, TE: Trou d'Eau

Low density sites: CAP: Cap-Méchant, PTE: Petit Trou d'Eau

S1_{cold}: austral cold season 2019, S2_{warm}: austral warm season 2020, S3_{cold}: austral cold season 2020

Clonal diversity of Stichopus chloronotus

On the 216 individuals of *S. chloronotus* genotyped, 166 presented no missing data. From them, 19 MLGs were identified, of which 9 were shared between 2 and 48 individuals. (**Supplementary material 7**). The numbering of the MLGs cited in this study is the one used in the previous study (Pirog *et al.*, 2017). Five MLGs seemed to be dominant: MLG34 was shared by 48 individuals, MLG05 was shared by 45, MLG02 by 22, MLG16 by 13 and finally MLG01 was shared by 15 individuals.

Table 5: Indices of genetic diversity and clonal structure for *Stichopus chloronotus* populations from Reunion Island

Season	Site	%NA	N	N_{MLG}	R	N_a	N_p	H_o	H_e	F_{IS}
S1	PAS	79.17	5	4	0.75	1.78 ± 0.22	0.11 ± 0.11	0.40 ± 0.13	0.28 ± 0.08	-0.44*** ± 0.12
	TE	66.67	8	3	0.29	1.78 ± 0.32	0.11 ± 0.11	0.17 ± 0.10	0.16 ± 0.06	-0.02 ^{NS} ± 0.21
	ES	33.33	16	6	0.33	1.67 ± 0.17	0.22 ± 0.15	0.38 ± 0.13	0.26 ± 0.07	-0.50*** ± 0.19
S2	PAS	4.17	23	7	0.27	1.78 ± 0.22	0.11 ± 0.11	0.25 ± 0.09	0.23 ± 0.07	-0.08 ^{NS} ± 0.12
	TE	12.50	21	3	0.10	1.78 ± 0.22	0.11 ± 0.11	0.12 ± 0.10	0.10 ± 0.05	-0.24** ± 0.24
	ES	0.00	24	2	0.04	1.56 ± 0.18	0.11 ± 0.11	0.41 ± 0.15	0.24 ± 0.08	-0.73*** ± 0.11
S3	PAS	8.33	22	6	0.24	2.00 ± 0.33	0.22 ± 0.15	0.26 ± 0.09	0.20 ± 0.06	-0.29*** ± 0.05
	TE	0.00	24	3	0.09	1.78 ± 0.22	0.00 ± 0.00	0.13 ± 0.10	0.12 ± 0.05	-0.02 ^{NS} ± 0.24
	ES	4.17	23	3	0.09	1.78 ± 0.15	0.11 ± 0.11	0.39 ± 0.12	0.26 ± 0.07	-0.50*** ± 0.10

%NA: percentage of missing data from the initially sampling design due to genotyping difficulty; N: number of individuals with no missing data; N_{MLG} : number of distinct multi-locus genotypes; R: clonal richness; N_a : mean number of alleles; N_p : mean number of private alleles; H_o and H_e : observed and expected heterozygosities respectively; F_{IS} : inbreeding coefficient and significant deviations from Hardy-Weinberg Equilibrium (**: $P < 0.01$; ***: $P < 0.001$; ^{NS}: non-significant). Standard errors are indicated following means values.

PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé

S1_{cold}: austral cold season 2019, S2_{warm}: austral warm season 2020, S3_{cold}: austral cold season 2020.

Clonal propagation of *Stichopus chloronotus* through space and time

Sampling design highlighted a spatial heterogeneity among sites in the distribution of *S. chloronotus* clones; each site was characterized by its own dominant clones (**Figure 6**), with no MLG shared between both reefs. However, clonal distribution was stable over the three seasons ($S1_{\text{cold}}$, $S2_{\text{warm}}$ and $S3_{\text{cold}}$) as, for a given site, the same MLGs were found for each season (**Figure 6**). The clonal richness was higher in $S1_{\text{cold}}$ for all the sites: 0.75 for PAS, 0.29 for TE and 0.33 for ES (**Table 5**), but it may be explained by the low number of individuals that correctly amplified during genotyping. It was also higher for each and over the three seasons at PAS (**Table 5**), which is dominated by two MLGs: MLG01 representing 28% of the individuals sampled at this site, and MLG02 representing 40% of the individuals (**Figure 6**). Over all seasons, only one dominant MLG was found at TE (MLG34 representing overall 46% of the individuals) and at ES (MLG05 representing overall 71% of the individuals ; **Figure 6**). For ES, in $S2_{\text{warm}}$, only two MLGs were found: MLG05 and MLG16, representing 83% and 17% of the individuals, respectively (**Figure 6**). The clonal richness was in consequence the lowest here (0.04; **Table 5**). PAS and TE, both located in the same reef complex, less than three kilometres apart, shared four MLGs: MLG01, MLG12, MLG34 and MLG37. No MLGs were shared between ES and the other sites, ES being located in another reef more than 20 kilometres southward (**Figure 6**). In conclusion, despite the spatial variability observed, there was no seasonal effect on the clonal distribution of *S. chloronotus*, nor any interannual effect during our monitoring; therefore, clonal propagation remained stable, as already found in (Pirog *et al.*, 2017).

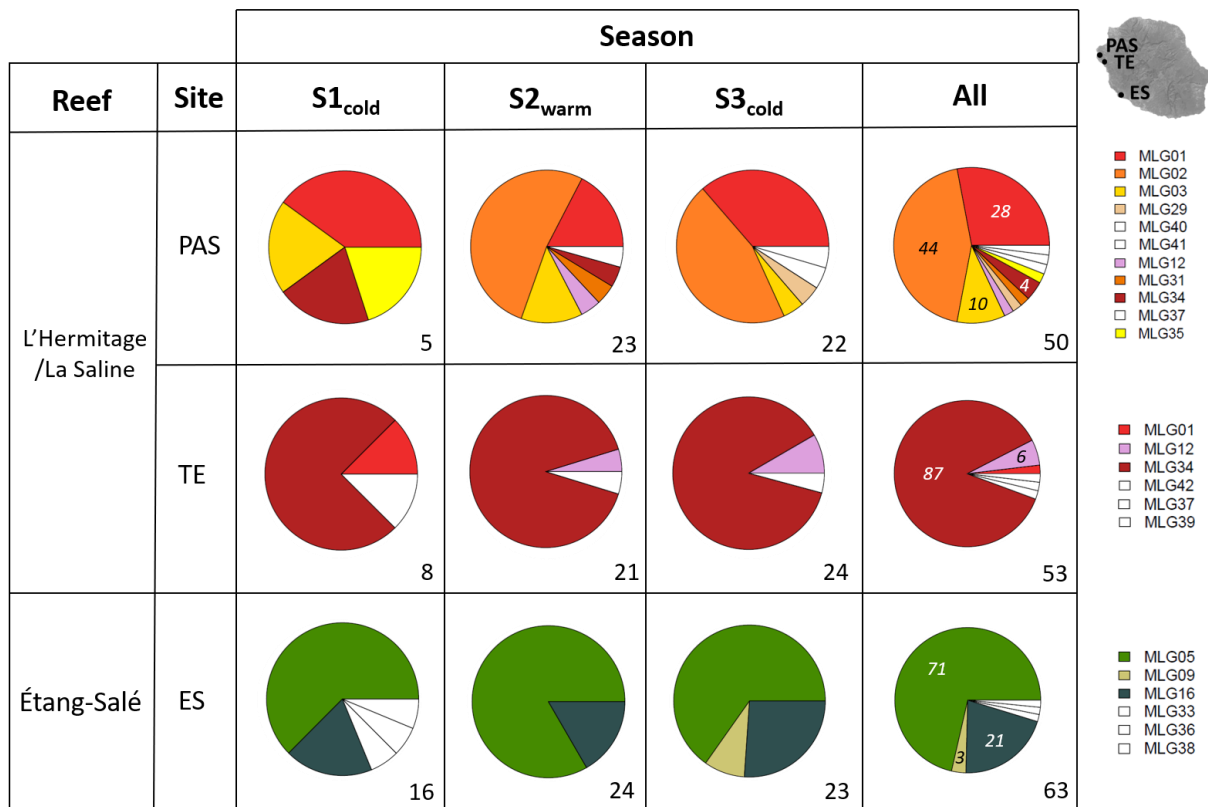


Figure 6: Spatial and temporal clonal distribution of *Stichopus chloronotus*

Numbers of individuals are indicated at the bottom right.

MLGs are coloured following Pirog et al. (2017). MLGs in white are unique over Pirog et al. (2017) and this study.

PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé

S1_{cold}: austral cold season 2019, S2_{warm}: austral warm season 2020, S3_{cold}: austral cold season 2020

Population structure and differentiation

The number of alleles per locus (N_a) and the number of private alleles (N_p) ranged between 0.29 ± 0.11 and 10.54 ± 0.72 for *H. leucospilota* (Table 4). On the contrary, N_a and N_p were very low and similar among all populations (site \times season) for *S. chloronotus*, ranging between 1.56 ± 0.18 and 2.00 ± 0.33 and between 0.00 ± 0.00 and 0.22 ± 0.15 , respectively (Table 5). The observed heterozygosity (H_o) and the expected heterozygosity (H_e) of *H. leucospilota* populations ranged between 0.39 ± 0.05 to 0.59 ± 0.04 and 0.79 ± 0.03 to 0.83 ± 0.02 , respectively and all sites deviated significantly from HWE (Table 4). For *S. chloronotus*, H_o and H_e ranged between 0.12 ± 0.10 to 0.41 ± 0.15 and 0.10 ± 0.05 and

0.28 ± 0.08, respectively, and almost all populations deviated significantly from HWE (**Table 5**).

Results from Structure and DAPC assignments at $K = 2$ were not congruent for *H. leucospilota* (**Figure 7**), indicating that there is no genetic structure among the five sites nor among seasons. Results of best K and BIC (**Supplementary material 8**) were also not congruent. These results were well supported by the pairwise F_{ST} calculated between pairs of populations (**Table 6**) where only few were significantly different from zero.

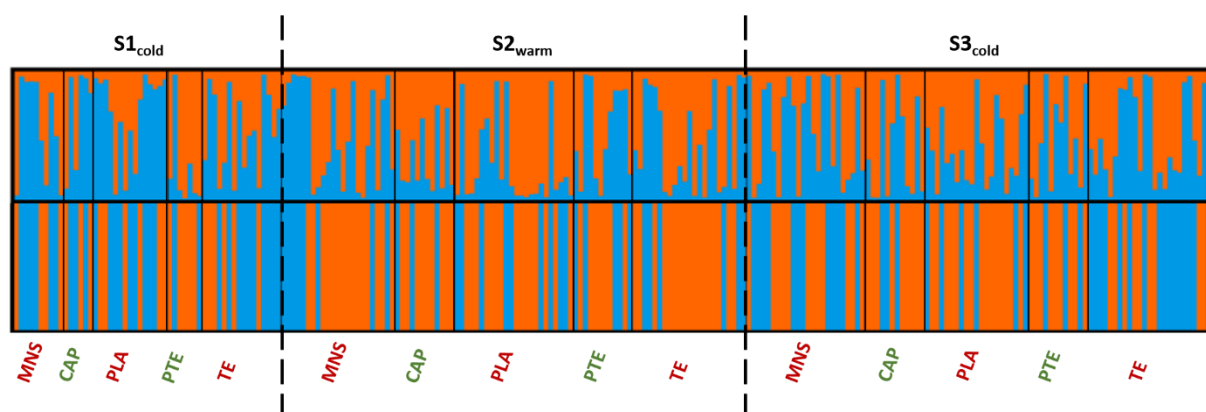


Figure 7: Assignment tests for *Holothuria leucospilota* individuals from the three seasons ($S1_{cold}$, $S2_{warm}$, $S3_{cold}$) on each site

Above, Structure plot at $K = 2$ and below, DAPC assignments at $K = 2$

High density sites in red: MNS: Maître-Nageur-Sauveteur, PLA: Planch'Alizé, TE: Trou d'Eau

Low density sites in green: CAP: Cap-Méchant, PTE: Petit Trou d'Eau

Table 6: Genetic differentiation of *Holothuria leucospilota* populations with all individuals kept estimated with Weir and Cockerham's F_{ST}

Season	Site	S1 _{cold}					S2 _{warm}					S3 _{cold}				
		MNS	CAP	PLA	PTE	TE	MNS	CAP	PLA	PTE	TE	MNS	CAP	PLA	PTE	TE
S1 _{cold}	MNS (10)	–														
	CAP (6)	0.007	–													
	PLA (15)	0.015	0.007	–												
	PTE (7)	0.033	0.028	0.017	–											
	TE (16)	0.020	0.040	0.035	0.039	–										
S2 _{warm}	MNS (23)	0.018	0.015	0.010	0.005	0.029*	–									
	CAP (12)	0.045	0.036	0.048*	0.027	0.066***	0.029*	–								
	PLA (24)	0.020	0.017	0.015	0.002	0.024	0.014	0.019	–							
	PTE (12)	0.033	0.021	0.026	0.006	0.045*	0.014	0.044***	0.012	–						
	TE (23)	0.042*	0.028	0.032*	0.012	0.040*	0.012	0.038***	0.013	0.009	–					
S3 _{cold}	MNS (24)	0.029	0.031	0.028*	0.009	0.042***	0.014	0.032*	0.016	0.007	0.013	–				
	CAP (12)	0.027	0.025	0.023	0.008	0.042	0.006	0.016	0.010	0.021	0.013	0.007	–			
	PLA (21)	0.026	0.015	0.012	0.009	0.045***	0.009	0.015	0.012	0.021	0.017	0.013	0.005	–		
	PTE (12)	0.035	0.031	0.023	0.002	0.045*	0.015	0.023	0.009	0.019	0.011	0.004	0.003	0.011	–	
	TE (24)	0.018	0.032	0.017	0.009	0.029*	0.008	0.044***	0.014	0.009	0.008	0.002	0.004	0.019*	0.008	–

Pvalues (*: $P < 0.5$; ***: $P < 0.001$) are indicated in bold.

High density sites in red: MNS: Maître-Nageur-Sauveteur, PLA: Planch'Alizé, TE: Trou d'Eau

Low density sites in green: CAP: Cap-Méchant, PTE: Petit Trou d'Eau

For each population, N is indicated in parentheses.

Even if some clones were detected, i.e., individuals from the same MLG or MLL assigned to the same cluster, results from Structure and DAPC assignments at $K = 2$ were not congruent for *S. chloronotus* (Figure 8), as well as the results of the best K and BIC (Supplementary material 9). However, keeping only one representative per MLG per site and pooling all seasons, DAPC showed a genetic differentiation between the reef of L'Hermitage/La Saline (PAS and TE) and the reef of Étang-Salé (ES) (Supplementary material 10) for *S. chloronotus* populations. Results of pairwise F_{ST} for *S. chloronotus* revealed that ES was significantly genetically different from PAS and TE for each season (Table 7). It is congruent with the absence of shared MLGs observed between these sites. Few significant differences were observed among seasons in PAS and TE (Table 7). Moreover, no significant genetic differentiation was observed among seasons for each site (Table 7).

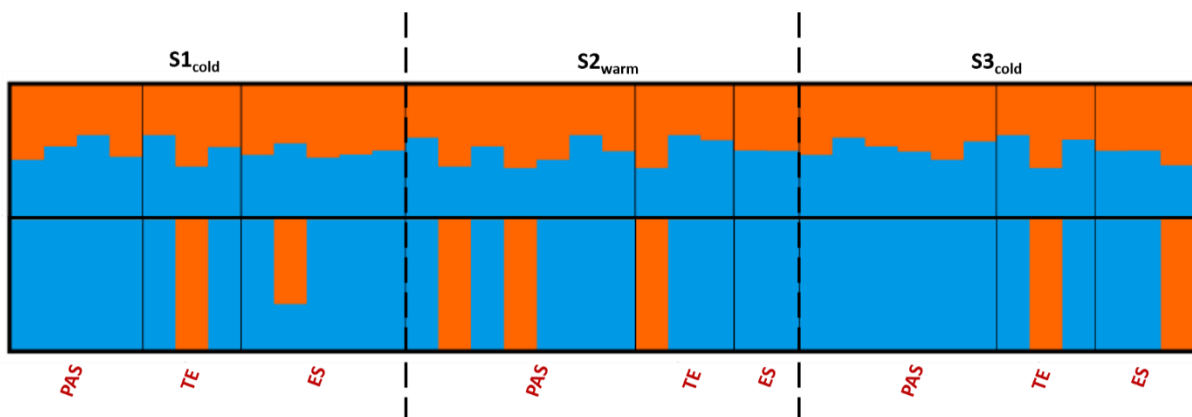


Figure 8: Assignment tests for *Stichopus chloronotus* individuals from the three seasons ($S1_{cold}$, $S2_{warm}$, $S3_{cold}$) on each site

Above Structure plot at $K = 2$ and below DAPC assignments at $K = 2$

PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé

Table 7: Genetic differentiation of *Stichopus chloronotus* populations with all individuals kept estimated with Weir and Cockerham's F_{ST}

Season	Site	S1 _{cold}			S2 _{warm}			S3 _{cold}		
		PAS	TE	ES	PAS	TE	ES	PAS	TE	ES
S1 _{cold}	PAS (5)	–								
	TE (8)	0.002	–							
	ES (16)	0.081**	0.113***	–						
S2 _{warm}	PAS (23)	-0.002	0.064	0.195***	–					
	TE (21)	0.147*	-0.016	0.220***	0.138***	–				
	ES (24)	0.105**	0.142***	-0.016	0.207***	0.237***	–			
S3 _{cold}	PAS (22)	0.017	0.068*	0.232***	-0.004	0.129***	0.240***	–		
	TE (24)	0.095	-0.032	0.188***	0.117**	-0.018	0.209***	0.111***	–	
	ES (23)	0.071*	0.115***	-0.020	0.189***	0.219***	-0.007	0.231***	0.188***	–

Pvalues (*: $P < 0.5$; **: $P < 0.01$; ***: $P < 0.001$) are indicated in bold

PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé

For each population, N is indicated in parentheses.

Discussion

Importance of the sexual reproduction for Holothuria leucospilota

Although both species exhibit the ability to reproduce sexually and asexually (Dolmatov, 2014), their main mode of reproduction seems different. Results on MLGs and MLLs clearly indicate that there is no clone among the populations of *H. leucospilota* at any site nor season. It looks surprising regarding a previous study in Reunion Island where individuals undergoing fission were observed (Conand *et al.*, 1997): a fission rate of 5.2% was estimated by a visual census in Trou d'Eau (TE, herein). This fission rate is low compared to those estimated for *H. atra* on the same reef, ranging between 14.9% and 19.6% (Conand, 1995, 2004). However, despite the high number of individuals undergoing fission estimated for *H. atra*, no clone was identified using microsatellite markers (Pierrat *et al.*, 2022c).

Several hypotheses may explain the absence of clones for *H. leucospilota*. First, only one study estimated the fission rate in *H. leucospilota* from Reunion Island (Conand *et al.*, 1997), dated upto 25 years, and no genetic study concerning the reproduction of this species has been realised since then. Moreover, the lifespan of sea cucumbers in their natural habitat

is a very problematic question, as no long-term capture-recapture method has yet been developed because of the rejection by tegument of any external tag (Gianasi *et al.*, 2015). For 25 years, sea cucumbers that have reproduced asexually may have died and sexual reproduction may have become the main mode of reproduction, leading to a high genetic diversity. Sexual reproduction of *H. leucospilota* seems to occur twice a year. The pattern of sexual reproduction in populations of *H. leucospilota* from Reunion Island has been investigated (Gaudron, Kohler & Conand, 2008), using gonad index and field observations. They observed that the first spawning event occurred in February and the second in May. Further studies found the same pattern for sexual reproduction of *H. leucospilota* in different localities, including Hong-Kong (Ong Che, 1990), Cook Islands (Drumm & Loneragan, 2005) and Heron Island (Great Barrier Reef) (Franklin, 1980). However, the spawning of *H. leucospilota* occurred in a short period of two weeks in April in Darwin (Australia) (Purwati & Luong-van, 2003). Therefore, the relative rate of sexual reproduction compared to asexual reproduction seems to be much higher given the low rate of fission previously estimated (Conand *et al.*, 1997) and the absence of clones in the population observed in this study. Sexual reproduction, favouring genetic mixing, could explain that individuals do not share MLG. Even the 1000 simulations, reducing genotyping to nine microsatellite markers over 24, showed that no MLGs were shared among individuals. As a comparison, a previous study did not find any shared MLGs in the *H. atra* population (Pierrat *et al.*, 2022c), for which the number of alleles was high and in the same order of magnitude as for *H. leucospilota* (48 for *H. leucospilota* and 42 for *H. atra*). As a consequence, our results showed that *H. leucospilota* populations of Reunion Island have not use asexual reproduction for a long period.

Results of Structure and DAPC and the low values of F_{ST} between population pairs suggest that populations of *H. leucospilota*, whatever the site density (high or low), are weakly or not genetically differentiated. Therefore, populations of *H. leucospilota* throughout the fringing reef of L'Hermitage/La Saline are actually a metapopulation with larval/gametes exchanges within the reef. Moreover, this low genetic differentiation among sites is also found among seasons, meaning that sexual reproduction in *H. leucospilota* is stable through time in this part of the world

Importance of asexual reproduction for Stichopus chloronotus

In contrast to *H. leucospilota*, individuals of *S. chloronotus* were grouped into few clones (only very few individuals presented a unique MLG). Asexual reproduction for *S. chloronotus* was already reported (Conand *et al.*, 1998), using a visual census for detecting whether some individuals underwent fission. They revealed that the fission rate reached 16% in Trou d'Eau (TE herein), and fell to 0% in Étang-Salé (ES herein). Once again, we showed that genetic tools, such as microsatellite markers, seem more consistent to study clonal propagation than fission rate estimated by a visual census. In fact, only 6 MLGs were identified at Trou d'Eau (TE herein) and 6 others at Étang-Salé (ES herein), which were shared between 53 and 63 individuals, respectively.

Other studies used genetic tools allowing a comparison of the percentage of individuals sharing MLGs. In our study, we found that 94% of the individuals sampled shared MLGs all sites and seasons combined, as in (Pirog *et al.*, 2017) which reported 97%. Analyses using allozymes revealed that 95% of the individuals on the Great Barrier Reef (Australia) shared MLGs (i.e., $R = 0.24$) (Uthicke, Benzie & Ballment, 1999). Using AFLP, 51 MLGs were identified within the 149 individuals sampled (i.e., $R = 0.34$), with up to 20 individuals presenting the same MLG (Uthicke & Conand, 2005). Overall, the percentage of individuals sharing MLG is very high for *S. chloronotus* populations from different localities, meaning that asexual reproduction seems to occur at a very high rate, higher than sexual reproduction, which would lead to a higher genetic diversity.

Our results showed that PAS and TE, in the same reef, shared some MLGs but none with ES, located in another reef, meaning that clonal propagation is limited to the reef-scale. The same pattern was already observed in Reunion Island for sea cucumbers (Pirog *et al.*, 2017) and for corals (Gélin *et al.*, 2017). However, differences in the dominant MLGs per site were observed through time, as in the previous study (Pirog *et al.*, 2017), except for PAS, where the same MLG (MLG02) was dominant. At ES, MLG05 is the MLG dominant in both studies, but MLG04, the previous second dominant MLG (Pirog *et al.*, 2017), was not identified in our study, replaced by MLG16, already found previously, but in few

individuals (Pirog *et al.*, 2017). The number of individuals sampled in both studies was different, with on average 64 and 24 individuals for the previous study (Pirog *et al.*, 2017) and our study respectively, due to a change of the density from $2.3 \pm 0.2 \text{ ind.m}^{-2}$ (Pirog *et al.*, 2017) to $0.7 \pm 0.1 \text{ ind.m}^{-2}$ (unpublished data). This decrease in density may have led to the loss of clones presenting a weaker fitness, explaining the variation in dominant MLGs observed between both studies. For TE, there is a clear shift of the dominant MLG between both studies; MLG01 was dominant between 2013 and 2016 (Pirog *et al.*, 2017), but it was only identified in one individual in our study, where MLG34 dominated.

Clonal propagation is stable through seasons. A previous study on clonal propagation of *S. chloronotus* from Reunion Island also showed no difference in the composition of MLGs within populations over four seasons (Pirog *et al.*, 2017). Asexual reproduction of *S. chloronotus* in Reunion Island reaches a maximum level in the end of austral winter in October, with the highest fission rate of about 24% (Conand *et al.*, 1998). More recently, in winter 2013, the fission rate of *S. chloronotus* in Reunion Island has quietly decreased and reached 11.5% (P. Frouin, unpublished data). The fission rate reached 31% in July for the population at the Great Barrier Reef (Australia) (Uthicke, 1997). Therefore, asexual reproduction occurs often in the cold season, where the environmental conditions are the less favourable, and sexual reproduction in the warm season (Uthicke, 1997; Conand *et al.*, 1998). As we found a temporal stability in the number of MLGs through seasons, sexual reproduction might occur but at very low rate, and asexual reproduction is the main mode of reproduction for *S. chloronotus* in Reunion Island.

Differences in reproductive strategies in two sympatric sea cucumber species

Sympatric species share the same biotic and abiotic conditions. Here, two sea cucumbers species, *H. leucospilota* and *S. chloronotus*, have a patchy distribution with high density in the reefs of Reunion Island. We showed that these two species, while both able to reproduce sexually and asexually through fission, tend to use distinct reproductive modes and above all, at different seasons: sexual reproduction through gamete spawning in the warm

season for *H. leucospilota* and clonal reproduction by transversal fission in the cold season for *S. chloronotus*.

Some studies on sea cucumbers have already shown that sympatric species that theoretically are able to reproduce asexually do not always do so (Bonham & Held, 1963; Franklin, 1980). For instance, *Holothuria atra* individuals underwent fission whereas *H. leucospilota* did not in Marshall Islands (Bonham & Held, 1963). Additionally, this difference in reproductive strategies has already been highlighted for other marine sympatric species, such as sea stars. For example, *Leptasterias hexactis* and *Pisaster ochraceus*, both sympatric in San Juan Island (USA), have two distinct strategies of reproduction: the first broods few and large youths in the brood chamber in winter, whereas the second broadcasts many small eggs in spring, reducing the interspecific competition for habitat and food resources (Menge, 1975). Moreover, the coral genus *Pocillopora* includes broadcast spawners and brooders, which can be frequently found in the same reef (Schmidt-Roach *et al.*, 2012).

Another reproductive strategy for reducing interspecific competition with the same reproductive mode is to alternate the period of reproduction. Analyses of gonadosomatic index revealed that two sympatric species of crabs in Guanabara Bay (Brazil) have a seasonal and alternative reproductive peak, with *Callinectes danae* reproducing in autumn and winter and *Callinectes ornatus* in spring and summer (Keunecke *et al.*, 2012). Authors concluded that the reproductive strategies of the two species of crabs leads to the avoidance of direct interspecific competition for available resources for planktonic larvae. Moreover, two sympatric species of sponges brood at two different times in the year, with *Dysidea avara* in June and July and *Phorbas tenacior* from August to October, avoiding overlap of the larval release period (de Caralt *et al.*, 2018).

Therefore, our study highlights that these two sympatric sea cucumber species from Reunion Island use different reproductive strategies at different periods of the year: asexual reproduction in the cold season for *S. chloronotus* and sexual reproduction in the warm season for *H. leucospilota*. This non-overlapping of reproductive periods helps to reduce the interspecific competition for both food resources and habitat space. In fact,

sexual reproduction leads to planktotrophic larvae, which migrate with the current, whereas asexual reproduction produces twice as many small individuals, but still in the adult stage, which are deposit-feeders, remaining in the same high-density patch into the reef. Additionally, even if post-settled sea cucumbers from sexual reproduction can be found near adult patches, they do not exhibit the same behaviour as adults and do not feed on the same food resources until they reach a specific size (Mercier, Battaglene & Hamel, 2000b).

Conclusion

This study highlights that two sympatric sea cucumber species from Reunion Island that have the ability to reproduce both sexually and asexually (by fission), each using one of these strategies of reproduction preferentially. *Holothuria leucospilota* reproduces sexually whereas *S. chloronotus* reproduces mainly asexually. Therefore, there is no overlap in the reproduction periods of the two species, as both modes of reproduction occur in different seasons. These two different strategies of reproduction drastically reduce the interspecific competition for food and habitat, in a context of hyperdensity. Knowledge on the ecology and genetic structure and diversity of these two sea cucumber species will be very useful for aquaculture purposes.

Statements & Declarations

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Competing Interests

The authors declare no conflict of interest

Author Contributions

Conceptualization, Joséphine Pierrat, Patrick Frouin and Hélène Magalon; Formal analysis, Joséphine Pierrat, Nicolas Oury and Hélène Magalon; Funding acquisition, Patrick Frouin and Hélène Magalon; Investigation, Joséphine Pierrat and Nicolas Oury; Project administration, Hélène Magalon; Supervision, Hélène Magalon; Validation, Joséphine Pierrat, Nicolas Oury and Hélène Magalon; Visualization, Joséphine Pierrat; Writing – original draft, Joséphine Pierrat; Writing – review & editing, Nicolas Oury, Patrick Frouin and Hélène Magalon.

Ethics approval

Individuals were collected thanks to the permission of Direction de l'Environnement, de l'Aménagement et du Logement (DEAL decision N°2020-37).

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Supplementary material

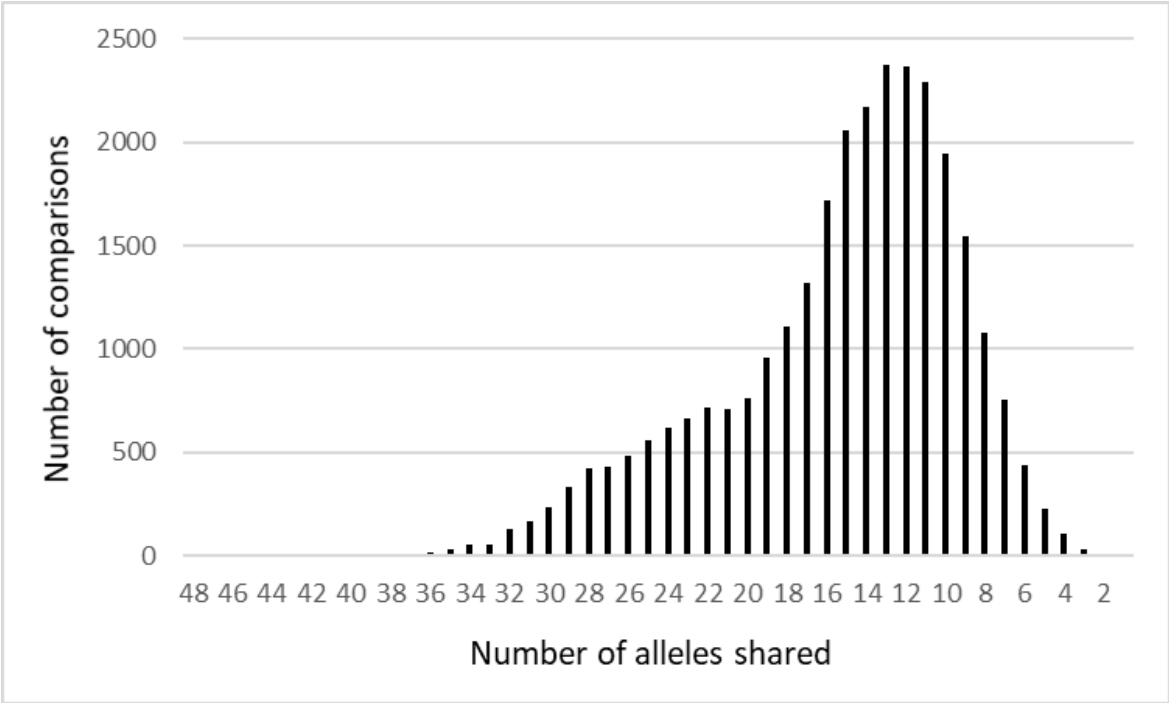
Supplementary material 5: Summary of the sampling design

Species	Reef	Site	Density	Code	Latitude (S)	Longitude (E)	N S1 _{cold}	N S2 _{warm}	N S3 _{cold}	N Total
		Maître-Nageur-Sauveteur	High	MNS	21°04'10.54"	55°13'11.92"	24	24	24	72
<i>Holothuria leucospilota</i>	L'Hermitage/La Saline	Cap-Méchant	Low	CAP	21°04'54.05"	55°13'23.29"	12	12	12	36
		Planch'Alizé	High	PLA	21°05'41.70"	55°14'01.65"	24	24	24	72
		Petit Trou d'Eau	Low	PTE	21°05'59.95"	55°14'25.73"	12	12	12	36
		Trou d'Eau	High	TE	21°06'11.52"	55°14'44.61"	24	24	24	72
<i>Stichopus chloronotus</i>	L'Hermitage/La Saline	Passe de l'Hermitage	High	PAS	21°05'05.58"	55°13'32.39"	24	24	24	72
		Trou d'Eau	High	TE	21°06'11.52"	55°14'44.61"	24	24	24	72
	Étang-Salé	Étang-Salé	High	ES	21°16'05.89"	55°19'57.29"	24	24	24	72

N indicates the number of individuals sampled.

S1_{cold}: austral winter 2019; S2_{warm}: austral summer 2020; S3_{cold}: austral winter 2020

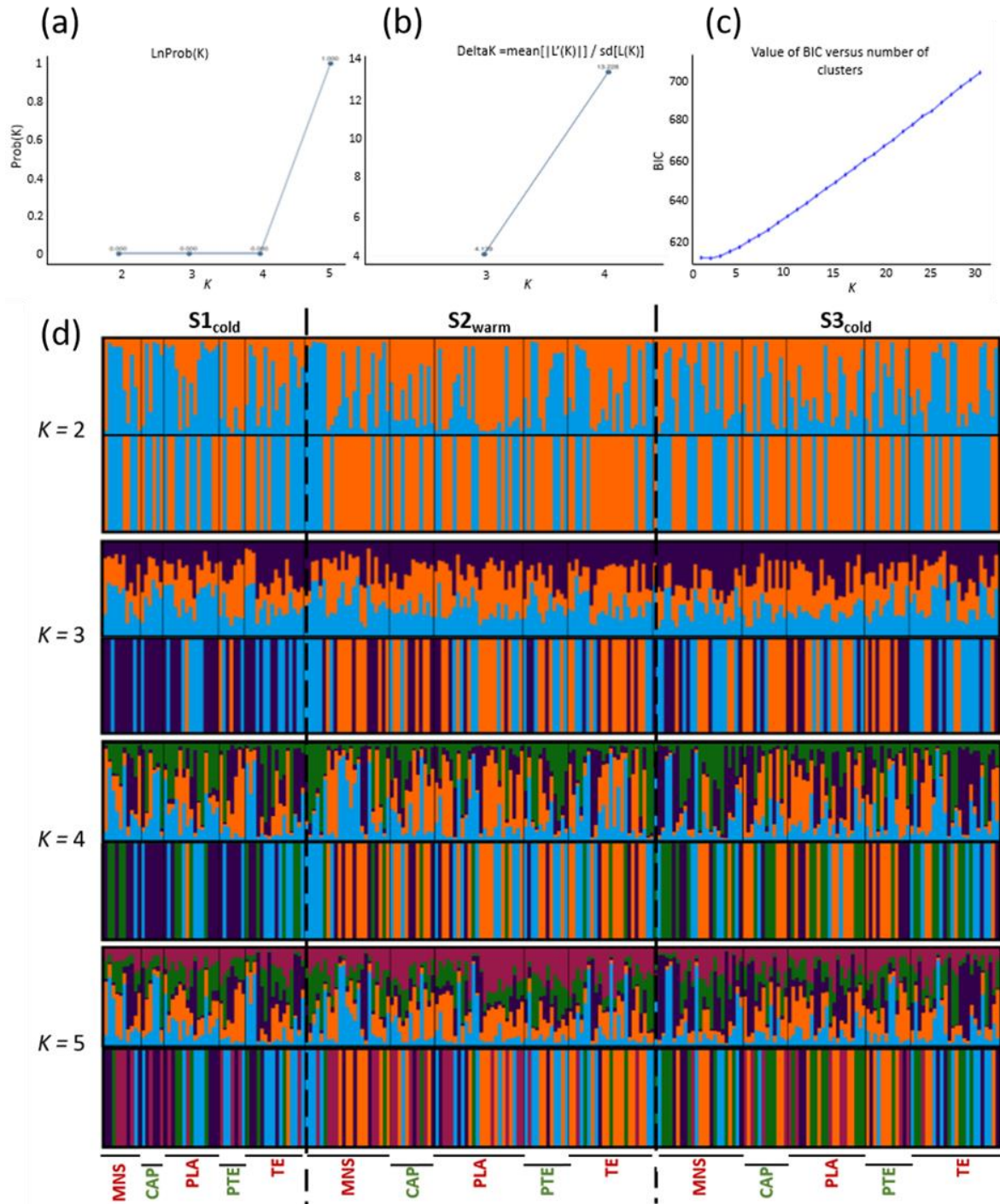
Supplementary material 6: Distribution of pairwise differences (number of alleles shared) among multi-locus genotypes (MLG) for *Holothuria leucospilota*.



Supplementary material 7: Number of individuals of *Stichopus chloronotus* per multi-locus genotypes (MLG) identified. The numbering of MLGs comes from Pirog et al. (2017).

MLG	Number of individuals
MLG34	48
MLG05	45
MLG02	22
MLG01	15
MLG16	13
MLG03	5
MLG12	4
MLG09	2
MLG37	2
MLG29	1
MLG31	1
MLG33	1
MLG35	1
MLG36	1
MLG38	1
MLG39	1
MLG40	1
MLG41	1
MLG42	1
Total	166

Supplementary material 8: Results of the assignment tests for *Holothuria leucospilota*. (a) mean likelihood over the five iterations of the same K, (b) Evanno's ΔK distribution, (c) BIC distribution and (d) plots from K = 2 to K = 5 for Structure (above) and DAPC (below).

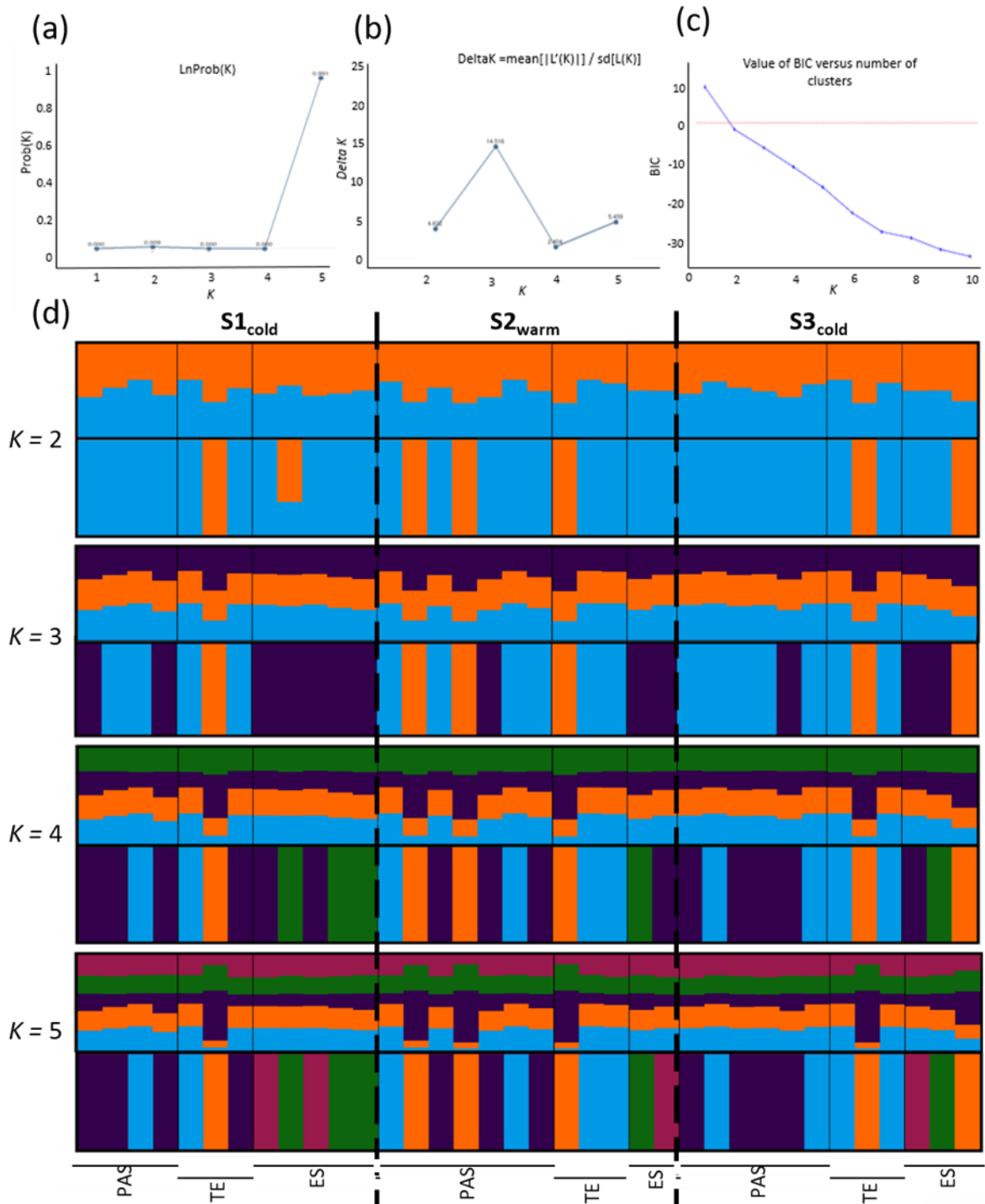


High density sites in red: MNS: Maître-Nageur-Sauveteur, PLA: Planch'Alizé, TE: Trou d'Eau

Low density sites in green: CAP: Cap-Méchant, PTE: Petit Trou d'Eau

S1_cold: austral cold season 2019, S2_warm: austral warm season 2020, S3_cold: austral cold season 2020

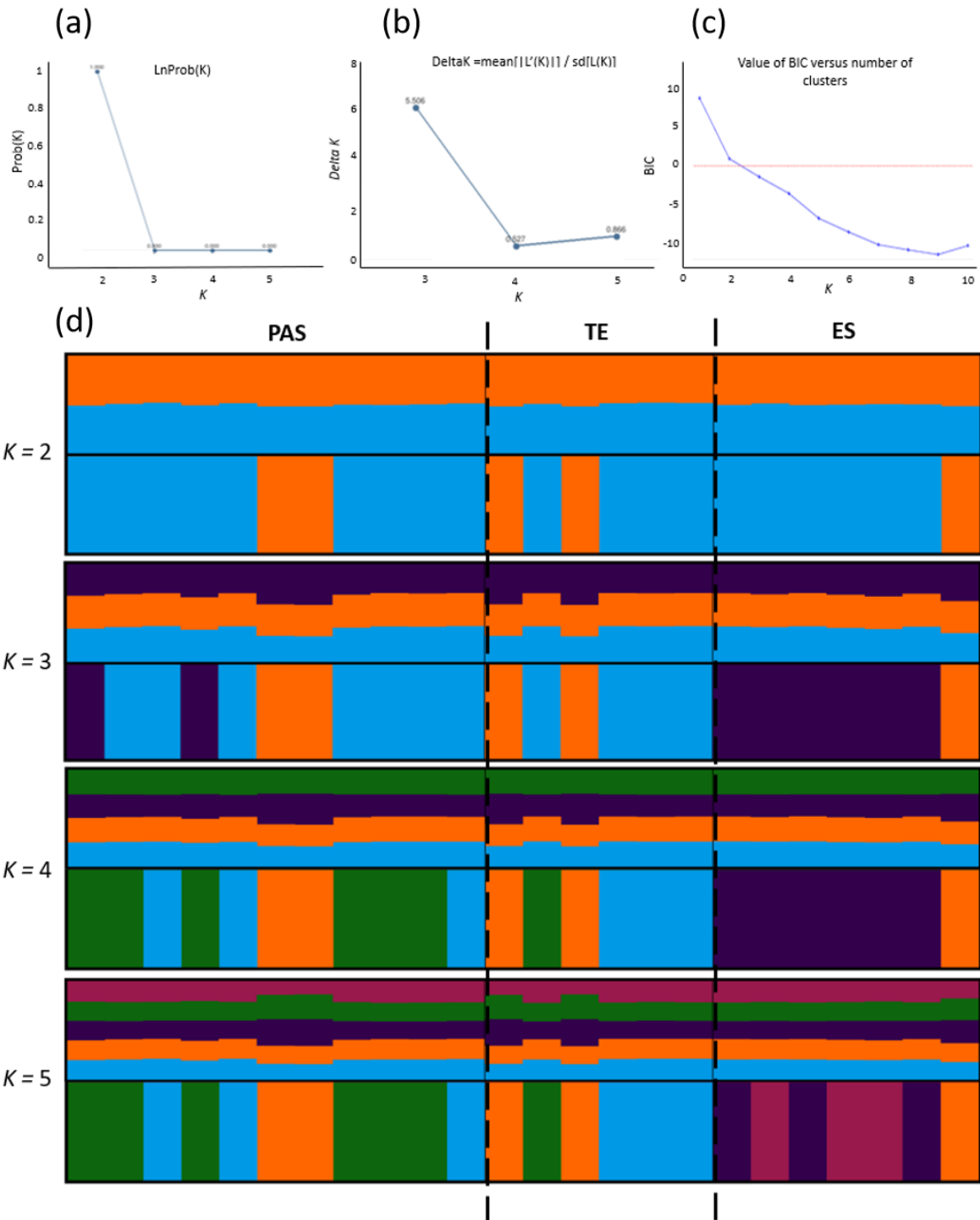
Supplementary material 9: Results of the assignment tests for *Stichopus chloronotus*. (a) mean likelihood over the five iterations of the same K, (b) Evanno's ΔK distribution, (c) BIC distribution and (d) plots from K = 2 to K = 5 for Structure (above) and DAPC (below).



PAS: Passe de l'Ermitage, TE: Trou d'Eau, ES: Étang-Salé

S1_{cold}: austral cold season 2019, S2_{warm}: austral warm season 2020, S3_{cold}: austral cold season 2020

Supplementary material 10: Results of the assignment tests for *Stichopus chloronotus* keeping only one representative per MLG, for each site, all seasons pooled. (a) mean likelihood over the five iterations of the same K, (b) Evanno's ΔK distribution, (c) BIC distribution and (d) plots from K = 2 to K = 5 for Structure (above) and DAPC (below).



PAS: Passe de l'Hermitage, TE: Trou d'Eau, ES: Étang-Salé

Chapitre 3 –

Variation spatio-temporelle des populations d'holothuries en lien avec leur environnement



*Individus de Holothuria leucospilota et Holothuria atra
dans la dépression d'arrière récif de La Saline*

16.02.2019

Résumé

Outre les facteurs génétiques, les facteurs environnementaux peuvent influencer la dynamique spatio-temporelle des holothuries et expliquer leur distribution, comme les facteurs sédimentaires, par exemple la concentration en matière organique ou en chlorophylle, les facteurs liés à la colonne d'eau, par exemple la salinité ou la température ou encore les facteurs physiques du milieu comme l'hydrodynamisme.

À La Réunion, plusieurs études font état de la densité exceptionnelle de trois espèces holothuries dans le récif de l'Hermitage/La Saline et de l'Étang-Salé, formant des agrégations. Il s'agit de *Holothuria atra*, *Holothuria leucospilota* et *Stichopus chloronotus*, parfois observé jusqu'à quatre individus par mètre carré. Ces densités sont considérées comme exceptionnelles pour des espèces d'holothuries dépositives de taille moyenne. Néanmoins, les facteurs influençant la distribution en agrégat de ces trois espèces n'ont pas été mis en évidence actuellement.

Ce chapitre vise à identifier les principaux facteurs régissant la dynamique spatio-temporelle des populations d'holothuries à forte densité à La Réunion et leur distribution en agrégation. Plus particulièrement, ce chapitre se concentre, en parallèle des populations d'holothuries, sur les facteurs sédimentaires tels que la composition en substrat, la matière organique, les particules fines, la chlorophylle *a*, et la signature isotopique en carbone et en azote.

Les résultats de ce chapitre sont soumis dans *Journal of Limnology & Oceanography*.

What drives the patchy distribution of tropical sea cucumbers? A multispecific monitoring study

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Abstract

Three species of sea cucumbers, *Holothuria atra*, *Holothuria leucospilota* and *Stichopus chloronotus*, are patchily distributed within coral reefs of Reunion Island. They exhibit high density ($> 1 \text{ ind.m}^2$). Factors involved in the distribution and spatio-temporal dynamics of these populations are not well understood yet. The aim of this study is to identify the key sedimentary factors which drive the dynamics of sea cucumbers populations of Reunion Island. Sea cucumber populations and sedimentary factors (substrates composition, total organic matter, fine particles, chlorophyll *a*, $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$) were monitored along a back-reef and shore-to-ocean gradient during four consecutive seasons. Meanwhile, feeding behaviour was assessed by measuring the motility and excretion rate. Multiseasonal monitoring revealed that the three species have their own dynamics, with an increase of density for *H. leucospilota*, a stability observed for *H. atra*, but a decrease for *S. chloronotus*, since 1990s. *Stichopus chloronotus* seems specialist while the two other generalists. Sedimentary factors with signs of anthropogenic disturbances (high organic matter, chlorophyll *a* and $\Delta^{13}\text{C}$) was related to *H. atra* distribution, while seagrasses variable was correlated with *H. leucospilota*. Conspecific attraction may drive the high-density patchy distribution of these two species. Finally, no sedimentary factor was correlated with *S. chloronotus*. The drastic diminution of the

populations of *S. chloronotus* could be linked to unstudied factors such as illegal harvesting, diseases, predation or climate change. Further studies should focus on other environmental factors in other reef compartments to fully explain the patchy distribution of sea cucumbers at Reunion Island.

Keywords

Sea cucumbers • Long term monitoring • Sedimentary factors • Specialist or opportunist species • Patchy distribution • Feeding behaviour • Reproductive modes

Introduction

Deposit-feeding sea cucumbers have been shown to play substantial ecological roles within shallow-water ecosystems and their impact on their associated environments has been recently reviewed by Purcell *et al.* (2016). Sea cucumbers are also remarkable taxa for human activities, such as (1) indicator species for monitoring pollution (Warnau *et al.*, 2006; Pierrat *et al.*, in submission), (2) medicines, with many active substances found in sea cucumbers tissues, such as antibacterial and antifungal (Adibpour *et al.*, 2014), antitumor and anticoagulant (Popov *et al.*, 2011), anticancer (Janakiram *et al.*, 2015), (3) food consumption, traditionally consumed raw, dried or boiled in most of Asian countries (Lovatelli *et al.*, 2004; Toral-Granda *et al.*, 2008). To face the high demand of the Asian market (Purcell, Williamson & Ngaluafe, 2018) and the collapse of most of the high-commercial value species worldwide (Friedman *et al.*, 2011), aquaculture processes have been developed (Yang *et al.*, 2015a; Hamel *et al.*, 2022). In consequence of their usefulness, interest for sea cucumbers has greatly increased since the 1990s (Pierrat *et al.*, 2022a).

Paradoxically, knowledge on the dynamics of wild populations and the influence of environmental factors is still sparse. Data on demographical and populational parameters are required to understand environmental factors driving populations of wild sea cucumbers. Even if sea cucumbers are easy to spot in shallow waters of coral reefs, monitoring methods are quite restricted, as individuals reject any tag within hours or weeks (Rodríguez-Barreras, Lopez-Morell & Sabat, 2016). Individual parameters, such as lifespan, age, or age of sexual

maturity are therefore impossible to estimate. Some species can reproduce asexually through transversal fission (Dolmatov, 2014; Pierrat *et al.*, 2022b, 2023), adding complexity. Local monitoring of wild sea cucumber populations is needed to fulfil knowledge gap and feed efficient management plans to preserve natural stocks from collapse, what is important for the health of reefs and for many human activities.

Some studies have underlined that few environmental factors could influence sea cucumbers. However, most of these studies have focused at the individual level, and not at the population level. Environmental factors followed during these studies were mostly measured during experiments *in vitro* and links with populations parameters were not realised. Jobson *et al.* (2021) showed that low salinity induced cellular (coelomocytes increased), hormonal (rise of cortisol levels) and behavioural (cloacal opening rates increased whereas force of attachment to the substrate decreased) shifts in *Cucumaria frondosa*. Hou *et al.* (2019) have exposed *Apostichopus japonicus* to desiccation and highlighted a shift of several enzymes with a decrease of glucose level, superoxide dismutase and catalase activities, while lactate, cortisol, malondialdehyde and oxygen species contents increased. Barkai (1991) found thanks to flow tank aquarium that sea cucumber species exhibited different ability to face water velocity. Bulteel, Jangoux & Coulon, (1992) evidenced that the depth was an important environmental factor driving the distribution of sea cucumbers according to size. Water temperature seems also influenced sea cucumbers, as Bonham & Held (1963) showed that the temperature threshold for *Holothuria atra* was 39°C, while *Holothuria leucospilota* was not feeding at 38°C. These studies underpin that a large range of environmental factors can influence the cellular, hormonal, behavioural processes and distribution of sea cucumbers, even if the link between populational and environmental parameters was missing.

Sea cucumbers inhabiting coral reefs are benthic organisms for which sedimentary compartment may play an essential role in their distribution. The relationship between sedimentary factors, including substrate composition, and sea cucumber density have been studied for wild sea cucumbers. Slater, Carton & Jeffs, (2010), Dissanayake & Stefansson (2012) and Navarro *et al.* (2013) attributed sea cucumbers density to sedimentary factors

linked to feeding, such as high organic matter or chlorophyll a loads, while Slater & Jeffs, (2010), Eriksson, Byrne & Torre-Castro (2012), Asha *et al.*, (2015), Tanita & Yamada, (2019) and Hamamoto *et al.* (2022) found correlations between type of substrates or habitats, such as coral rubbles or seagrasses, and sea cucumber density.

Almost 40 tropical species of sea cucumbers are found in coral reefs of Reunion Island (Conand *et al.*, 2018). Few studies have assessed their abundance since 1990. They highlighted that three deposit-feeding species have a patchy distribution across reefs with very high densities: *H. leucospilota*, *H. atra* and *Stichopus chloronotus* (Conand *et al.*, 1998; Jaquemet *et al.*, 1999; Cuvillier, 2016), which have the ability to reproduce both sexually and asexually (Dolmatov, 2014). Such heterogeneous distribution with high density spots of sea cucumbers has already been reported (Shiell & Knott, 2010; Hernández-Flores *et al.*, 2018). At some locations within Reunion Island reefs, two of the species could be found together at high densities (Conand & Mangion, 2002). Those studies did not investigate the link between environmental and populational parameters.

The aim of this study is to assess the spatio-temporal variability of patchily distributed sea cucumber populations in a small reef complex at Reunion Island, and identify the sedimentary factors leading to high-density patches, with consideration of the feeding patterns.

Materials & Methods

Study area and species studies

Study was conducted in the volcanic island of Reunion Island, which is a French department, located in the Mascareignes Archipelago in the South Western Indian Ocean (21°06'S, 55°31'E). The tropical climate of Reunion Island has two seasons: austral summer, described as warm and humid, and austral winter, considered as cold and dry (Conand *et al.*, 2007). Dating back to three million years (Duncan *et al.*, 1989), Reunion Island possesses four fringing reefs on the West (Hermitage/La Saline, Saint-Leu and Étang-Salé reefs) and South (Saint-Pierre reef) coasts. Some locations within the reefs received nutrient inputs from

enriched terrestrial waters, and are therefore considered as eutrophic (PAS, PLA, TE and ES sites, see next sections for details, Cuët, Naim & Faure, 1988; Tedetti *et al.*, 2011; Cuvillier, 2016). Two monitoring were realised to investigate back reef and shore-to-ocean variabilities.

Inter-site variability: back-reef gradient

Four sites at the back-reef depression (**Figure 9**) were chosen considering their high densities ($> 1 \text{ ind.m}^2$) for the three sea cucumber species of this study. From the North to the South in Hermitage/La Saline reef: (1) “MNS” site (Maître-Nageurs-Sauveteurs), is situated in the narrowness part of the reef (150 m), then subject to intense swells. Monospecific seagrass meadows of *Syringodium isoetifolium* are present at this site and home high-density population of *H. leucospilota* (Cuvillier, 2016). (2) “PAS” site (Passe de l’Hermitage) is in the middle of the reef of Hermitage/La Saline. A high-density population of *S. chloronotus* characterises the site (Cuvillier, 2016). (3) TE site (Trou d’Eau) is located in the south part, La Saline. High-density populations of *H. leucospilota* and *S. chloronotus* have been recorded at this site (Conand & Mangion, 2002). Finally, (4) ES site (Étang-Salé), is the only one located at the Étang-Salé reef, with high-density population of *S. chloronotus* (Conand *et al.*, 1998).

Intra-site variability: shore to ocean gradient

“PLA” site (Planch’Alizés) is located in the south part of Hermitage/La Saline reef (**Figure 9**). High-density populations of *H. leucospilota* and *H. atra* have been registered at this site (Conand & Mangion, 2002). However, a different spatial pattern for *H. atra* close to the shore and *H. leucospilota* at the edge of the back-reef depression, near the inner reef flat was observed. In this sense, an adapted sampling was carried out at this site, with a shore-to-ocean gradient composed by three stations: PLA_{SHORE}, PLA_{MID} and PLA_{OCEAN}.

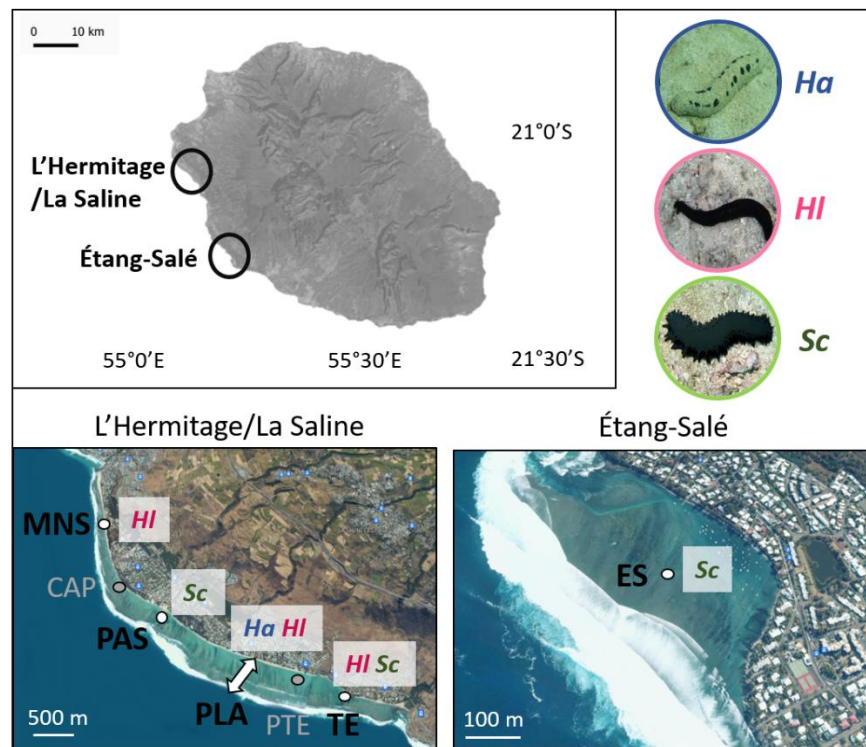


Figure 9: Location of Reunion Island in the western Indian Ocean. Location of L'Hermitage/La saline and Étang-Salé reefs on the west coast of Reunion Island, and sampling sites at each reef.

HI : *Holothuria leucospilota* ; *Ha* : *Holothuria atra* ; *Sc* : *Stichopus chloronotus*

Sampling design

Population metrics

Densities at each site were estimated with four belt-transects of 50 × 2 m, bimonthly from August 2019 to January 2021 for the back-reef gradient. Regarding the shore-to-ocean gradient at PLA site, three belt-transects of 50 × 2 m were drawn at each of the three stations (PLA_{SHORE}, PLA_{MID} and PLA_{OCEAN}), from July 2020 to July 2022. Densities of all species was recorded at each site, and biometric variables (width × length) were measured on the first 30 individuals of each species in each transect.

Sedimentary factors

Quadrats sizing 2 × 2 m were plotted every two meters along the belt-transects in order to estimate substrates composition. Ten categories of substrates were observed *in situ*: 'sand', 'seagrasses', 'sponges', 'cyanobacteria', 'red macroalga', 'living coral without turf', 'living coral with turf', 'erected dead coral', 'coral rubble without turf', and 'coral rubble with

turf'. Additionally, sediment samples were cored (core 15 cm² x 4.5 cm) in triplicates at each sites and stations for sediment analyses: (1) quantification of organic matter load, (2) fine particles (< 63 µm), (3) chlorophyll *a*, and (4) determination of $\Delta^{15}\text{N}$ and $\Delta^{13}\text{C}$. Sediment control samples were also collected in triplicates at two low-density sites (< 0.001 ind.m², CAP and PTE) for three consecutive seasons (Winter 2019, Summer 2020 and Winter 2020).

Feeding features

Speed and excretion rate were used as proxies to characterize the feeding behaviour of each species. The moving speed of sea cucumbers was estimated at each site for each species. In the field, 15 individuals were tagged, thanks to plastic dermal T-implant on the dorsal tegument. At T= 0 min, locator stakes were placed behind the posterior end of each individual. Every 15 min, during two hours, the distance between the posterior end of the individual and the locator stake was measured. The locator stakes were replaced behind individual posterior end every 15 min. In order to highlight a possible specific active moving period for searching food, this experiment was repeat at 8 and 11 a.m. and 2 p.m. The tags were recovered at the end. The moving speed has been calculated for each individual.

The assessment of the ingestion rate is impossible *in situ*, therefore the excretion rate, which is a proxy of the amount of sediment ingested, was estimated. Faeces were collected inside five quadrats of 1 × 1 m at each site and separated by species. The density inside the quadrats was also recorded. This experiment was repeat at 8 and 12 a.m. and 4 p.m. to highlight a possible diel pattern. Night activity was not considered. Ingestion rate is expressed in dry weight of sediment per individual per day. Moving speed and ingestion rate were assessed at MNS, PAS, PLA and TE sites.

Analyses of sediment and faeces samples

Calcination method was used to determine the total organic matter load (Byers, Mills & Stewart, 1978). First, sediment samples were dried 48h at 60°C and weighted to obtain dry weight. Sediment samples were then calcinated 4h at 450°C and weighted for ash free dry weight. The difference between the two weights gives the total organic matter load. Dry

weight was also quantified for particles analyses. The dried samples were then sieved on 63 µm mesh and dried a second time 48h at 60°C to obtain dry weight without fine particles.

Sediment samples for $\Delta^{15}\text{N}$ and $\Delta^{13}\text{C}$ analyses were freeze-dried during 48h. Then ground using a ball mill (3 min at 3,000 rpm). Sediment samples are naturally enriched in carbonates, which can alter isotopic analyses and acidification is often required (Kolasinski, Rogers & Frouin, 2008). Therefore, they were acidified with 1 ml of hydrochloric acid 1 N until no further CO_2 releases were observed (Jacob *et al.*, 2005). Sediment samples were then frozen (-18°C), freeze-dried again, encapsulated in silver and tin capsules, and sent to the isotopic spectrometry platform of LIENSs (Littoral Environment Societies, La Rochelle, France).

Before 90% acetone extraction overnight at 5°C (Lorenzen, 1966), sediment samples were freeze-dried during 48h for chlorophyll *a* analyses as recommended by Hansson (1988). The supernatant was removed and centrifuged 10 min at 3,000 rpm. A first spectrometry read was performed at 665 and 750 nm. Then, 0.45 µl of HCl was added to dissolve chlorophyll *a* pigments during 3 min (Holm-Hansen & Riemann, 1978). Finally, a second spectrometry read was realised. Samples were kept away from light with aluminium foil during the experiment. The concentration of chlorophyll *a* was expressed as:

$$[Chla] \text{ mg. L}^{-1} = \frac{26.7 \times [(A_{665}^{na} - A_{750}^{na}) - (A_{665}^a - A_{750}^a)]}{L}$$

with 26.7 the chlorophyll coefficient, A^{na} and A^a the absorbance before and after acidification at 665 and 750 nm, respectively, and L the optical path length (cm). Finally, the quantity of chlorophyll *a* was determined as: $Q_{chla} \text{ mg. m}^{-2} = [Chla] \times \frac{V}{S}$ with V the volume of acetone used for extraction (L) and S the core surface (0.0015 m²).

Faeces were dried 48h at 60°C and weighted. The density recorded in the quadrat, allowed to determine the excretion rate, which is a proxy of the ingestion rate (g.DW.ind⁻¹). Reported to the global density in each site, the bioturbation rate was calculated.

Statistical analyses

All numerical analyses were realized using R (R Core Team, 2022).

Population, sedimentary and feeding parameters

For all the analyses, the monthly replicates have been grouped into four seasons, following statistical analyses (P-value > 0.05 for months of each season): winter 2019, summer 2020, winter 2020 and summer 2021. Population variables (density and length of individuals) and feeding parameters (moving speed and excretion rate) were tested for spatial, temporal and specific variability using ANOVAs or PERMANOVAs. Additionally, two-way ANOVAs were used to determine spatio-temporal variabilities of the grouped sedimentary factors investigated during this study (see section below). For all analyses, post-hoc Tukey HSD or pairwise-PERMANOVA tests were used when necessary.

Sedimentary factors driving sea cucumber populations

Multiple linear regressions were built for both back-reef and shore-to-ocean gradients to highlight the sedimentary variables that mostly drive the sea cucumber density, at different spatial scales. Additionally, one sedimentary variable can have various effect on each species, therefore, one multiple linear regression was built for each species in each gradient. In order to reduce the number of sedimentary variables to build the models, standardized PCA was used and correlation tests between variables were investigated. In this sense, some sedimentary variables were clustered together regarding the significant correlation (mean correlation threshold= 37%, P-value < 0.05, see **Supplementary material 11**) and the ecological relevance. Therefore, a new variable 'dead coral' includes 'erected dead coral' and 'coral rubble without turf', a variable 'living erected organisms' includes 'sponges', 'living coral without turf' and 'living coral with turf', and a new variable 'algociano' includes 'cyanobacteria' and 'red macroalga'. Additionally, some other variables were removed for each gradient due to their significant high degree of correlation with other variables (mean correlation threshold= 52%, P-value < 0.001, see **Supplementary material 11**).

For the back-reef gradient, the calculated variable is expressed by the following equation for *H. leucospilota* and *S. chloronotus*:

$$\text{Density} = \text{season} + \text{station} + \text{chlorophyll } a + \text{sand} + \text{seagrasses} + \text{dead coral} + \text{living erected organisms} + \text{coral rubble with turf} + \text{chlorophyll } a \times \text{season} + \text{chlorophyll } a \times \text{station} + \text{sand} \times \text{season} + \text{sand} \times \text{station} + \text{seagrasses} \times \text{station} + \text{living erected organisms} \times \text{station} + \text{coral rubble with turf} \times \text{season} + \text{coral rubble with turf} \times \text{station} + \text{dead coral} \times \text{season} + \text{dead coral} \times \text{station}.$$

For the shore-to-ocean gradient in PLA, the calculated variable is expressed by the following equation for *H. atra* and *H. leucospilota*:

$$\text{Density} = \text{season} + \text{station} + \text{organic matter load} + \text{sand} + \text{fine particles} + \text{erected dead coral} + \text{living erected organisms} + \text{coral rubble with turf} + \text{algocyano} + \Delta^{13}\text{C} + \text{sand} \times \text{season} + \text{fine particles} \times \text{season} + \text{organic matter load} \times \text{station} + \text{season} \times \text{organic matter load} + \text{coral rubble with turf} \times \text{season} + \Delta^{13}\text{C} \times \text{station} + \Delta^{13}\text{C} \times \text{season}.$$

The *stepAIC* function in the R package MASS (Venables & Ripley, 2002) was used to find out the best models for each species of each gradient, which are presented in **Supplementary material 12**. Significant variables were tested with ANOVA for each final model, and adapted post-hoc tests were performed when necessary (Tukey HSD or correlation test).

Density over long-term monitoring

Raw density data of sea cucumbers were extracted from: (1) four published papers, Conand *et al.*, 1998; Jaquemet, Rousset & Conand, 1999; Conand & Mangion, 2002; Conand, 2004, (2) three masters reports, Mangion, 2003; Burgos & Hollinger, 2007; Pierrat, 2018, (3) two thesis, Cuvillier, (2016) and Bédier 2013 (unpublished data). PERMOVAs were performed to tests the temporal evolution of the density for each species at each site. Pairwise permutation t-tests were used when necessary.

Results

Population features

The distribution of length for the three species are shown in **Supplementary material 13**. For all sites and seasons combined the mean length of *H. leucospilota* reached 23.7 ± 8.1 cm (min= 3 ; max= 57), of *H. atra* 13.8 ± 4.8 (min= 3 ; max= 33) and of *S. chloronotus* 10.9 ± 5.2 (min= 1.5 ; max= 30). **Supplementary material 13** clearly highlights the absence of small lengths class sizes.

Inter-site analyses: back-reef gradient

Significant spatial variabilities for density (ANOVA, $N= 156$, $df= 3$, $F= 73.05$, P -value < 0.001) and length (ANOVA, $N= 156$, $df= 1$, $F= 81.91$, P -value < 0.001) of *H. leucospilota* were recorded. Two sites had high-density populations for this species: MNS, with the highest density but the smallest individuals (2.6 ± 0.2 ind.m⁻², 18.8 ± 0.4 cm) and TE with the longest individuals (1.2 ± 0.1 ind.m⁻², 24.1 ± 0.4 cm). No individual of *H. leucospilota* could be found at ES during our monitoring. Additionally, no temporal (intra-seasonal or seasonal) or inter-site spatial variability was observed for density and length of *H. leucospilota*.

The density of *S. chloronotus* was significantly different among sites (ANOVA, $N= 156$, $df= 3$, $F= 24.13$, P -value < 0.001), with the highest density recorded at TE (1.2 ± 0.1 ind.m⁻²), no difference between PAS and ES (0.8 ± 0.1 and 0.7 ± 0.1 ind.m⁻²) and no individuals observed at MNS. A temporal trend shows decreasing densities over the study period, with less individuals in 2021 (**Figure 10**). except for TE site with steady density. For PAS (ANOVAs, $N= 35$, $df= 3$, $F= 3.28$, P -value = 0.036 and $F= 16.45$, P -value < 0.001 , respectively), density and length of individuals were inversely correlated, with the highest density and the smallest individuals observed in Winter 2019 (1.1 ± 0.2 ind.m⁻², 7.9 ± 0.3 cm) and the lowest density with the longest individuals in Summer 2021 (0.5 ± 0.1 ind. m⁻², 15.2 ± 1.1 cm), multiplying the length/density ratio by more than 4 in 18 months only. Length of individuals was significantly different (ANOVA, $N= 156$, $df= 2$, $F= 23.45$, P -value < 0.001), with individuals longer at PAS and TE (11.6 ± 0.5 and 12.5 ± 0.7 cm, respectively) than at ES (7.8 ± 0.3 cm). No common trend appeared for length variation of *S. chloronotus* over the study period. Individual length

increased at PAS and TE (ANOVAs, $N= 35$, $df= 3$, $F= 16.45$, $P\text{-value} < 0.001$ and $N= 42$, $df= 3$, $F= 6.67$, $P\text{-value} = 0.001$) but decreased at ES (ANOVA, $N= 42$, $df= 3$, $F= 18.49$, $P\text{-value} < 0.001$) from Winter 2019 to Summer 2021 (Figure 10).

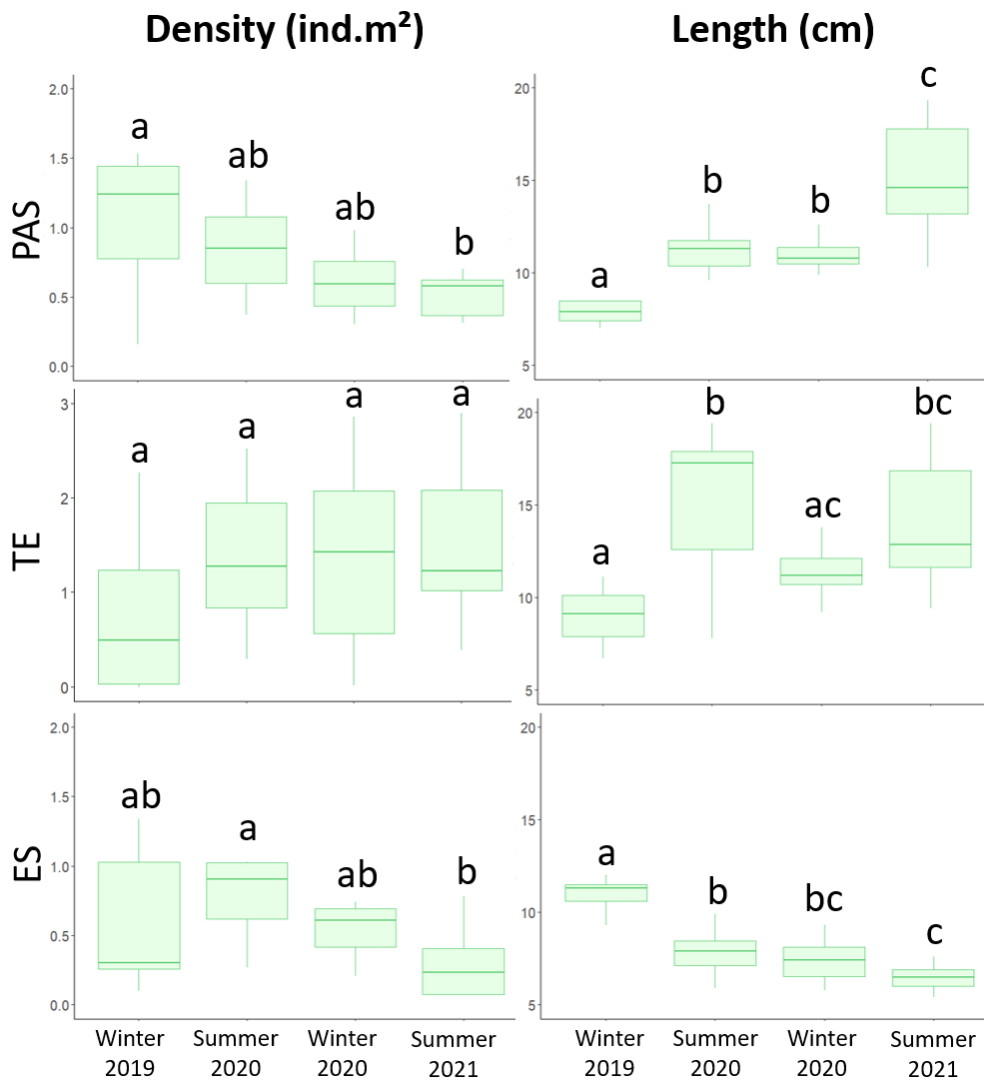


Figure 10: Temporal variability of the density (ind.m⁻²) and length (cm) of *S. chloronotus* at different site.

Different letters indicate significant differences (Tukey HSD, $P\text{-value} < 0.05$).

Intra-site variability: shore to ocean gradient for community

Significant spatial differences for density and length of *H. leucospilota* (ANOVAs, $N= 63$, $df= 2$, $F= 14.99$, $P\text{-value} < 0.001$ and $F= 25.73$, $P\text{-value} < 0.001$) and also for *H. atra* (ANOVA, $N= 63$, $df= 2$, $F= 53.1$, $P\text{-value} < 0.001$ and $F= 18.54$, $P\text{-value} < 0.001$) were observed at PLA (Figure 11). The highest density of *H. atra* was recorded at PLA_{SHORE} ($4.3 \pm 0.4 \text{ ind.m}^{-2}$) and the lowest at PLA_{OCEAN} ($0.3 \pm 0.0 \text{ ind.m}^{-2}$). The opposite pattern was observed for *H. leucospilota*, with highest densities at PLA_{MID} and PLA_{OCEAN} (0.8 ± 0.1 and $1.0 \pm 0.1 \text{ ind.m}^{-2}$, respectively) and the lowest at PLA_{SHORE} ($0.3 \pm 0.1 \text{ ind.m}^{-2}$). For both species, the longest individuals were found at PLA_{OCEAN} (30.9 ± 0.9 and $17.7 \pm 0.7 \text{ cm}$ for *H. leucospilota* and *H. atra*, respectively), and the smallest at PLA_{SHORE} (24.3 ± 0.6 and $12.3 \pm 0.3 \text{ cm}$).

No temporal variability (intra or inter-seasonal, nor at each site) was found for the density and length of *H. leucospilota*. However, the density and length of *H. atra* presented site effect for seasonal variability. At PLA_{SHORE}, the highest density was recorded in Summer 2021 ($5.0 \pm 0.4 \text{ ind.m}^{-2}$) and the lowest in Winter 2020 ($3.0 \pm 0.5 \text{ ind.m}^{-2}$). The opposite pattern was found at PLA_{MID}, with $3.3 \pm 0.6 \text{ ind.m}^{-2}$ in Winter 2020 and $1.0 \pm 0.3 \text{ ind.m}^{-2}$ in Summer 2021. Finally, at PLA_{MID} and PLA_{OCEAN}, *H. atra* individuals were longer in Summer 2021 (16.7 ± 1.2 and $19.5 \pm 1.2 \text{ cm}$) than in Winter 2020 (11.4 ± 0.5 and $14.1 \pm 0.4 \text{ cm}$).

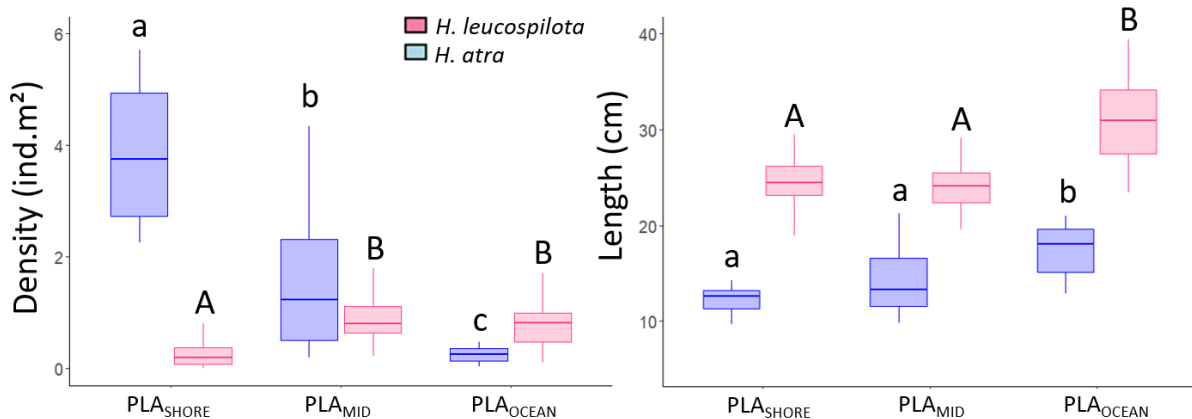


Figure 11: Temporal variability of the density (ind.m^{-2}) and length (cm) of *H. atra* (in blue) and *H. leucospilota* (in pink) over the shore to ocean gradient at PLA. Different letters indicate significant differences (Tukey HSD, $P\text{-value} < 0.05$).

Sedimentary patterns

Results of two-ways ANOVAs in **Table 8**, highlighted spatial heterogeneity for all sedimentary variables and seasonal significant differences for 8 out of 11.

A difference in substrates composition was observed between the two reefs, with Étang-Salé mainly dominated by three components: sand, coral rubble with turf and living erected organisms (**Figure 12**). Substrates at Étang-Salé were homogeneous across seasons, mostly dominated by living erected organisms and coral rubble with turf (**Figure 4**). Additionally, the sediment features at Étang-Salé were very particular, with the lowest total organic matter load ($2.01 \pm 0.03\%$), chlorophyll *a* concentration ($93.5 \pm 6.7 \text{ mg.m}^{-2}$), $\Delta^{15}\text{N}$ and $\Delta^{13}\text{C}$ ($-2.78 \pm 0.03\text{‰}$ and $-20.4 \pm 0.70 \text{‰}$ respectively), but the highest fine particles ($1.25 \pm 0.07\%$) (**Supplementary material 14**).

Sites in Hermitage/La Saline reef (MNS, PAS, PLA and TE) had more diverse substrates, with stronger presence of dead corals and seagrasses (at MNS only). Except for seagrasses, living erected organisms and $\Delta^{15}\text{N}$, all components presented significant seasonal variability (sand, algociano, coral rubble with turf, dead corals, total organic matter load, fine particles, chlorophyll *a* and $\Delta^{13}\text{C}$), without particular pattern (**Supplementary material 14**). Concerning the intra-site shore-to-ocean gradient at PLA, spatial variability was found only for total organic matter load (higher at $\text{PLA}_{\text{SHORE}}$ with $2.62 \pm 0.05\%$), and $\Delta^{13}\text{C}$ (higher at $\text{PLA}_{\text{SHORE}}$, $0.09 \pm 0.05\text{‰}$), as post-hoc test did not find any significant difference between sites for coral rubble with turf (the ANOVA significativity was very low, 0.049) (**Table 8**), meaning organic matter load and quality is heterogeneous there. Concerning substrates variables, a spatial homogeneity among stations in PLA was observed. Temporal variability was recorded for sand and coral rubble with turf on the three stations ($\text{PLA}_{\text{SHORE}}$, PLA_{MID} and $\text{PLA}_{\text{OCEAN}}$), with higher cover by coral rubble with turf in Summer 2021 while sand cover is higher in winter (**Figure 12**, **Supplementary material 15**). Organic matter load and $\Delta^{15}\text{N}$ were higher in Summer 2021 ($2.62 \pm 0.02\%$ and $4.12 \pm 0.05\text{‰}$, respectively), whereas $\Delta^{13}\text{C}$ was lower ($-0.19 \pm 0.02\text{‰}$).

Comparisons of sedimentary factors between low and high-density sites, from Winter 2019 to Winter 2020, revealed that low and high-density patches were homogeneous except for the Étang-Salé reef. In fact, organic matter load ranged between 1.95 ± 0.05 at ES and

$2.82 \pm 0.37\%$ at PLA_{SHORE} . Significant spatial differences were found between ES and all other sites (pairwise-PERMANOVA, $N= 69$, P -values < 0.05) except MNS. Percentage of fine particles, ranging from 0.19 ± 0.05 at PLA_{SHORE} to 1.16 ± 0.29 at PAS, was homogeneous between reefs (PERMANOVA, $N= 69$, $maxT= 2.252$, P -values = 0.213). Chlorophyll a concentration reached $75.4 \pm 17.2 \text{ mg.m}^{-2}$ at ES and was twice fold higher at TE, $156.0 \pm 23.9 \text{ mg.m}^{-2}$. Differences were found between ES and TE, ES and PTE, TE and CAP (pairwise-PERMANOVA, $N= 69$, P -values < 0.05). Almost each site had its own isotopic signature for $\Delta^{13}C$ (min. at ES -18.30 ± 2.38 and max. at PLA_{SHORE} $0.14 \pm 0.09\text{‰}$) and $\Delta^{15}N$ (min. at ES -2.42 ± 1.42 and max. at TE $5.82 \pm 0.29 \text{‰}$) (PERMANOVA, $N= 69$, $maxT= 7.147$ and 5.036 respectively, P -values < 0.001)

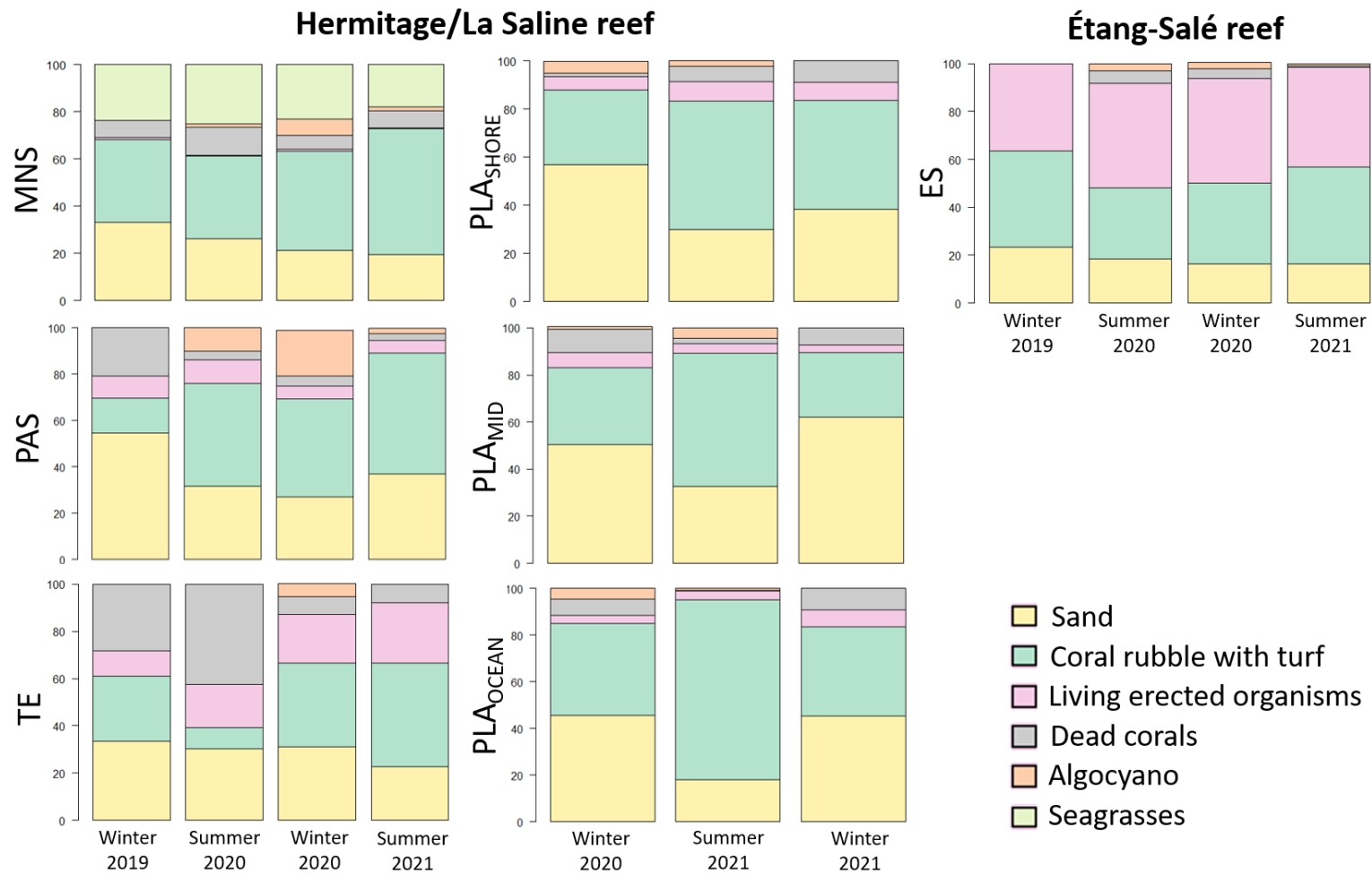


Figure 12: Substrates cover (in %) across the sites of the study over the seasons of monitoring.

Table 8: Two-ways PERMANOVA results of sedimentary parameters at the back-reef (MNS, PAS, TE and ES) and shore to ocean (PLA) gradient overs sites and seasons

	Back-reef gradient					Shore-to-ocean gradient				
	SS	Df	MS	F value	P-value	SS	Df	MS	F value	P-value
Sand										
Season	2666	3	888.7	5.702	< 0.001	8097	2	4048	14.468	< 0.001
Site	6576	3	2192.1	14.064	< 0.001	1654	2	827	2.955	0.061
Residuals	21821	149	155.9			15110	54	280		
Seagrasses										
Season	72	3	24	0.96	0.414					
Site	14386	3	4795	190.64	< 0.001					
Residuals	3521	149	25							
Algocyano										
Season	1923	3	641.1	8.517	< 0.001	117.6	2	58.79	2.09	0.134
Site	1421	3	473.7	6.292	< 0.001	5.1	2	2.56	0.091	0.913
Residuals	10539	149	75.3			1518.8	54	28.13		
Living erected organisms										
Season	58	3	19	0.188	0.905	7.7	2	3.87	0.213	0.809
Site	37618	3	12539	122.686	< 0.001	92.2	2	46.08	2.543	0.088
Residuals	14309	149	102			978.3	54	18.12		
Coral rubble with turf										
Season	6795	3	2265.1	8.549	< 0.001	10953	2	5476	15.664	< 0.001
Site	2902	3	967.4	3.651	0.014	2231	2	1115	3.19	0.049
Residuals	37092	149	264.9			18878	54	350		
Dead corals										
Season	3595	3	1198.3	6.08	< 0.001	321	2	160.31	2.61	0.083
Site	7962	3	2654	13.467	< 0.001	21	2	10.55	0.172	0.843
Residuals	27590	149	197.1			3316	54	61.42		
Organic matter load										
Season	0.766	3	0.2553	15.675	< 0.001	0.4698	2	0.23492	10.11	< 0.001
Site	7.902	3	2.6339	161.699	< 0.001	0.2936	2	0.14678	6.317	0.003
Residuals	2.28	149	0.0163			1.2548	54	0.02324		
Fines particles										
Season	33.37	3	11.122	99.587	< 0.001	15.631	2	7.815	41.57	< 0.001
Site	2.58	3	0.859	7.69	< 0.001	0.862	2	0.431	2.293	0.111
Residuals	15.64	149	0.112			10.152	54	0.188		
Chlorophyll a										
Season	18756	3	6252	7.142	< 0.001	1932	2	966	1.054	0.356
Site	56240	3	18747	21.414	< 0.001	4027	2	2013	2.197	0.121
Residuals	122560	149	875			49496	54	917		
$\Delta^{13}C$										
Season	107	3	36	5.582	0.001	1.2016	2	0.6008	59.294	< 0.001
Site	10001	3	3334	521.725	< 0.001	0.4847	2	0.2423	23.916	< 0.001
Residuals	895	149	6			0.5472	54	0.0101		
$\Delta^{15}N$										
Season	11.5	3	3.8	1.586	0.195	55.57	2	27.784	369.044	< 0.001
Site	1876.5	3	625.5	257.969	< 0.001	0.33	2	0.164	2.177	0.123
Residuals	339.5	149	2.4			4.07	54	0.075		

Motility and feeding patterns

Concerning the moving speed, no intraday significant difference was found for *H. leucospilota* and *H. atra* at each site (**Figure 13**). Conversely, a daily pattern was observed for *S. chloronotus* at PAS and TE, with a lower motility in the early morning, with 10.2 ± 4.7 and 32.5 ± 6.8 $\text{cm}\cdot\text{h}^{-1}$ respectively. Some spatial variability is shown for the motility of *H. leucospilota* only, which is higher at MNS (18.8 ± 2.4 $\text{cm}\cdot\text{h}^{-1}$) than at PLA and TE (pairwise-PERMANOVA, $N=135$, $\text{maxT}=4.267$, $P\text{-value} < 0.001$). Interspecific comparison for high-density species at the same site, gives (1) significantly higher speed for *S. chloronotus* (PERMANOVA, $N=90$, $Z= -6.489$, $P\text{-value} < 0.001$), 41.2 ± 3.4 $\text{cm}\cdot\text{h}^{-1}$ at TE, than *H. leucospilota* with 8.6 ± 1.1 $\text{cm}\cdot\text{h}^{-1}$ (**Figure 13**) and (2) similar speeds for *H. atra* and *H. leucospilota* at PLA (12.8 ± 2.1 and 8.9 ± 1.6 $\text{cm}\cdot\text{h}^{-1}$ respectively at PLA, PERMANOVA, $N=90$, $Z= 1.443$, $P\text{-value} = 0.149$).

Globally, the ingestion rate (**Figure 13**), had the same pattern as speed, except for *H. leucospilota*, which had the highest ingestion rate at TE compared to *S. chloronotus* (11.2 ± 1.9 and 4.53 ± 1.07 $\text{gDW}\cdot\text{ind}^{-1}\cdot\text{d}^{-1}$ respectively; PERMANOVA, $N=60$, $Z= 2.760$, $P\text{-value} = 0.006$).

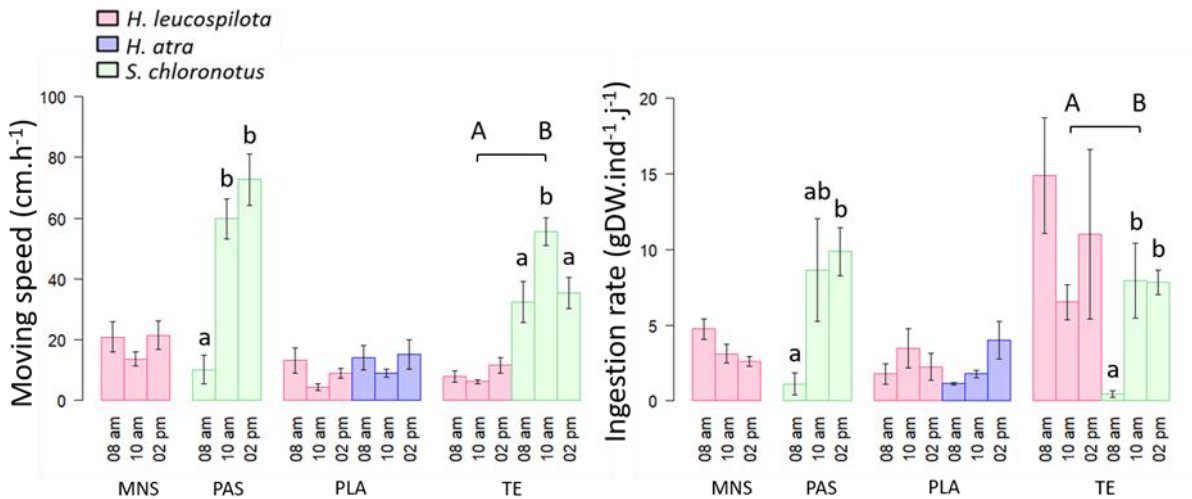


Figure 13: Moving speed ($\text{cm}\cdot\text{h}^{-1}$) and ingestion rate ($\text{gDW}\cdot\text{ind}^{-1}\cdot\text{d}^{-1}$) of *H. leucospilota* (in pink), *H. atra* (in blue), *S. chloronotus* (in green) in different times of the day (08 and 10 am, 02 pm) at different sites.

Different letters indicate significant differences (Pairwise Permutation Test, $P\text{-value} < 0.05$).

Sedimentary factors influencing sea cucumber densities

Results of the linear model for each species of the back-reef gradient are shown in **Table 9**. A significant positive correlation was found between the density of *H. leucospilota* and the seagrass coverage (P-value < 0.001, $r = 0.554$). Results of the model also showed that the substrates coral rubble with turf, erected living organisms and dead corals have a significant influence on the distribution of *H. leucospilota* in L'Hermitage/La Saline reef. While, for *S. chloronotus*, the interaction between season and chlorophyll a and season and 'dead coral' seem to have an effect on the density and distribution.

Regarding the shore-to-ocean gradient at PLA, no sedimentary variable was significant for *H. leucospilota* density, while significant positive correlation between the density of *H. atra* and the coverage of erected living organisms (P-value = 0.007, $r = 0,334$) existed. Moreover, 'season × total organic matter load' effects were found, with significant positive correlation between total organic matter load and density of *H. atra*, in Summer 2021 (P-value < 0.001, $r = 0.611$) and in Winter 2021 (P-value = 0.011, $r = 0.586$). Additionally, a 'site × total organic matter load' with significant correlation between total organic matter load and density at PLA_{SHORE} (P-value = 0.032, $r = 0.468$).

Table 9: Significance of individuals and ecological variables of each model (ANOVA results).

Back-reef gradient	SS	Df	MS	F value	P-value
<i>Stichopus chloronotus</i>					
Site	28.034	3	9.345	28.448	< 0.001
Season	2.002	3	0.667	2.032	0.113
Chlorophyll <i>a</i>	0.295	1	0.295	0.898	0.345
Dead coral	1.046	1	1.046	3.183	0.077
Coral rubble with turf	0.948	1	0.948	2.885	0.092
Season × Chlorophyll <i>a</i>	3.814	3	1.271	3.870	0.011
Season × Coral rubble with turf	1.78	3	0.593	1.806	0.149
Season × Dead coral	3.501	3	1.167	3.552	0.016
Residuals	42.375	129	0.329		
<i>Holothuria leucospilota</i>					
Site	157.671	3	52.557	100.940	< 0.001
Chlorophyll <i>a</i>	0.492	1	0.492	0.945	0.333
Seagrasses	4.509	1	4.509	8.661	0.004
Dead coral	1.917	1	1.917	3.682	0.057
Living erected organisms	1.204	1	1.204	2.313	0.131
Coral rubble with turf	4.632	1	4.632	8.895	0.003
Site × Chlorophyll <i>a</i>	3.944	3	1.315	2.525	0.061
Site × Living erected organisms	6.433	3	2.144	4.118	0.008
Site × Coral rubble with turf	3.425	3	1.142	2.192	0.092
Site × Dead coral	10.928	3	3.643	6.996	< 0.001
Residuals	66.126	127	0.521		
Shore- to- ocean gradient					
<i>Holothuria atra</i>					
Station	174.186	2	87.093	136.070	< 0.001
Season	0.769	2	0.385	0.601	0.553
Organic matter load	0.044	1	0.044	0.069	0.794
Sand	1.397	1	1.397	2.182	0.147
Fine particles	1.049	1	1.049	1.639	0.207
Dead coral	0.061	1	0.061	0.095	0.759
Living erected organisms	5.125	1	5.125	8.007	0.007
Coral rubble with turf	0.009	1	0.009	0.014	0.905
Algocyano	16.489	1	16.489	25.762	< 0.001
$\Delta^{13}\text{C}$	1.304	1	1.304	2.038	0.160
Season × Organic matter load	16.886	2	8.443	13.191	< 0.001
Site × Organic matter load	23.588	2	11.794	18.426	< 0.001
Site × $\Delta^{13}\text{C}$	3.518	2	1.759	2.748	0.075
Residuals	28.163	44	0.640		
<i>Holothuria leucospilota</i>					
Station	5.624	2	2.812	15.182	< 0.001
Season	0.031	2	0.016	0.085	0.919
Sand	0.019	1	0.019	0.103	0.750
Fine particles	0.022	1	0.022	0.120	0.731
Dead coral	0.012	1	0.012	0.066	0.798
Coral rubble with turf	0.039	1	0.039	0.212	0.647
$\Delta^{13}\text{C}$	0.075	1	0.075	0.405	0.527
Season × Fine particles	0.810	2	0.405	2.185	0.123
Site × $\Delta^{13}\text{C}$	1.168	2	0.584	3.154	0.051
Residuals	9.076	49	0.185		

Long-term dynamics for density

Back-reef gradient

The density of *H. atra* was very low and stable at the four sites MNS, PAS, TE and ES (**Figure 14**). An opposite trend was observed for *H. leucospilota* and *S. chloronotus*. Globally, the density of *H. leucospilota* seemed to increase through time on the two high-density sites, MNS and TE (**Figure 14**). In fact, at MNS, its density has doubled between the studies by Cuvillier (2016), with $1.6 \pm 0.1 \text{ ind.m}^{-2}$ and ours, with $3.1 \pm 0.5 \text{ ind.m}^{-2}$ in Summer 2021. Moreover, at TE, the density was multiplied by 10, from 0.2 ± 0.1 and $0.1 \pm 0.1 \text{ ind.m}^{-2}$ following Conand & Mangion (2002) and Mangion (2003) respectively, to 1.2 ± 0.2 for our study. At PAS and ES, the density was stable through time for this species. Conversely, a decreasing global trend was observed for the density of *S. chloronotus* (**Figure 14**). At PAS, even if no significant difference was found, a decreasing trend was reported with $0.9 \pm 0.4 \text{ ind.m}^{-2}$ for Bédier (2013) and successively 1 ± 0.2 , 0.8 ± 0.1 , 0.7 ± 0.1 and $0.5 \pm 0.0 \text{ ind.m}^{-2}$ during our monitoring. The same trend was observed at TE, with the highest density recorded by Conand *et al.* (1998) and Bédier (2013), 3.7 ± 0.6 and $3.8 \pm 0.4 \text{ ind.m}^{-2}$ respectively, and a significantly lower value found during our monitoring, with $1.3 \pm 0.3 \text{ ind.m}^{-2}$. Finally, at ES, decreasing density was highlighted for *S. chloronotus*, with $2.0 \pm 0.0 \text{ ind.m}^{-2}$ by Burgos & Hollinger (2007) and $0.3 \pm 0.1 \text{ ind.m}^{-2}$ in Summer 2021 (this study).

Shore to ocean gradient at PLA

The density of *H. atra* had decreased between Jaquemet *et al.* (1999), with $4.8 \pm 0.2 \text{ ind.m}^{-2}$, and Mangion (2003) with $3.2 \pm 0.6 \text{ ind.m}^{-2}$ (**Figure 15**). Such high densities were only recorded at PLA_{SHORE} during this study, with 4.5 ± 0.8 and $5.0 \pm 0.4 \text{ ind.m}^{-2}$ for Summer and Winter 2021 respectively. However, the mean density of *H. atra* reached $2.1 \pm 0.4 \text{ ind.m}^{-2}$ over sites and seasons, meaning that the general trend is still decreasing. The opposite pattern was found for *H. leucospilota*, with a general increasing trend (**Figure 15**), from a mean of $0.4 \pm 0.2 \text{ ind.m}^{-2}$ by Conand & Mangion (2002) and Mangion (2003) to 1.0 ± 0.2 and $0.8 \pm 0.2 \text{ ind.m}^{-2}$ at PLA_{MID} and PLA_{OCEAN} for this study.

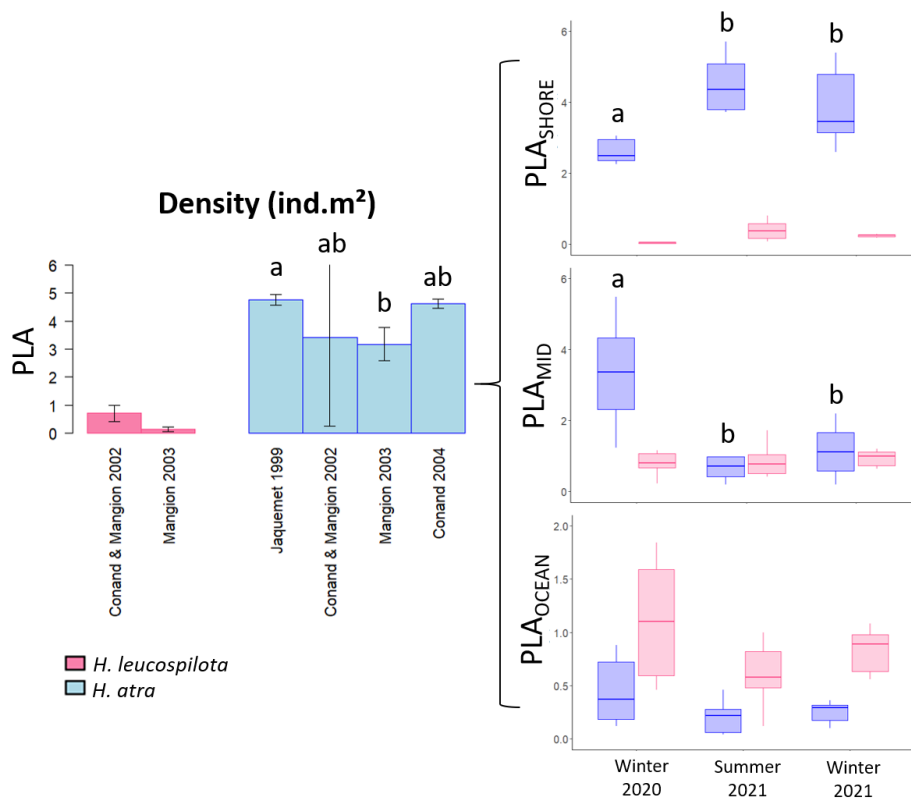


Figure 15: Long term comparison of the density (ind.m⁻²) of *H. leucospilota* (in pink) and *H. atra* (in blue) at PLA, and within the gradient sites of PLA (PLA_SHORE, PLA_MID and PLA_OCEAN). Different letters indicate significant differences (Tukey HSD, P-value < 0.05).

Discussion

Patchy distribution related to sedimentary factors in an anthropogenic context

Holothuria atra got only one dense patch within the reefs of Reunion Island, at PLA site. More precisely, this species exhibited very high density (about 4 ind.m²) close to the coast, at PLA_{SHORE} station. No difference for substrate cover (sand, coral rubble with turf, living erected organisms, dead corals and alcyonians) were observed along the shore-to-ocean gradient, meaning that the patchy distribution of *H. atra* at Reunion Island does not seem to be related to the substrate composition. Some studies have linked the patchy distribution of *H. atra* to substrates. Eriksson *et al.* (2012) and Asha *et al.* (2015) have correlated the distribution of *H. atra* with the presence of boulders and rubbles, but Tanita & Yamada (2019), Viyakarn *et al.* (2020) and Hamamoto *et al.* (2022) with sand. PLA_{SHORE} is the second station

(considering also the sites) where both sand (more than 40%) and coral rubble with turf (almost 45%) dominated but no correlation was highlighted for this study. In consequence, *H. atra* exhibits variability in substrate preference or its distribution is related to other factors not assessed during these studies. Some other sedimentary factors characterized the PLA_{SHORE} station, as the higher total organic matter load ($2.62 \pm 0.05\%$) and higher $\Delta^{13}\text{C}$ content ($0.09 \pm 0.05\text{‰}$) among all stations and sites, and the higher chlorophyll *a* concentration ($112.00 \pm 7.90 \text{ mg.m}^{-2}$) among PLA stations. This result is in agreement with those of Dissanayake & Stefansson (2012), Moriarty (1982) and Viyakarn *et al.* (2020), who respectively demonstrated that distribution of *H. atra* was correlated with organically enriched sediments and high chlorophyll *a* concentrations. Finally, the substrate type seems to have low impact on the distribution of *H. atra* compared to the quality of the organic matter or the presence of microalgae. These peculiar sedimentary factors may be influenced by anthropogenic inputs from terrestrial waters. Tedetti *et al.* (2011) revealed that the La Saline reef, where PLA site is located, is subject to anthropogenic contamination by sewage discharges and polycyclic aromatic hydrocarbons. Consequently, the patchy distribution of *H. atra* could also be linked to the high anthropogenic disturbance of the reef of La Saline, especially near the shore where effects of such pollution seem to be the most discernible. Conand & Mangion (2002) also showed that high level of eutrophication, leading to high organic matter availability, was suitable for populations of *H. atra*.

Correlation was found between the distribution of *H. leucospilota* and the presence of seagrasses, however this has to be considered carefully as seagrasses thrive only in one site. The distribution of *S. chloronotus* was unrelated to sedimentary factors. Some studies have investigated the link between sedimentary factors and the distribution of these two species. Hamamoto *et al.* (2022) observed that *H. leucospilota* was correlated with rubble and seagrass meadows whereas *S. chloronotus* was correlated with dead corals, scleractinian corals and coralline algae. The distribution of *H. leucospilota* has also been related to bedrock (Tanita & Yamada, 2019). Additionally, as the comparison of total organic matter, chlorophyll *a*, fine particle, $\Delta^{13}\text{C}$ and $\Delta^{15}\text{N}$ contents between low and high-density sites showed no differences, all these factors do not appear to control the distribution of these two species. Therefore, despite the high number of sedimentary factors monitored during this study, they do not seem

the most adapted for understanding and explaining the high-density patchy distribution of these two species.

As for *H. leucospilota* and *S. chloronotus* no usual sedimentary factors could explain their patchy distribution, other types of factors have to be considered. Among them, conspecific attraction has been observed in many marine taxa, such as tropical seabirds (Szostek, Schaub & Becker, 2014), fish (Lecchini, Planes & Galzin, 2007) crabs (Donahue, 2006) or echinoderms such as ophiuroids (Morgan & Jangoux, 2004), sea urchins or sea stars (Campbell *et al.*, 2001). Such behaviour has been attributed to predator protection, supportive habitat, synchronised reproduction or availability of food (Campbell *et al.*, 2001; Morgan & Jangoux, 2004; Donahue, 2006; Lecchini *et al.*, 2007). However, information is sparse concerning conspecific attraction for sea cucumbers. Recently, Marquet *et al.* (2018) have conducted *in vitro* experiments to understand the chemical communication of *Holothuria arguinensis* during spawning aggregations. They found that males release pheromones, possibly composed of phosphatidylcholines that induces both males and females spawning, even for other phylogenetically close species as *Holothuria mammata*. A very recent study has discovered a pheromone, composed by disaccharide saponins, that are secreted by sea cucumbers surrounded by quality and available food (Claereboudt *et al.*, 2023). Authors observed that starved individuals did not secreted this pheromone anymore. This secretion induces a positive chemotaxis in conspecifics, and in consequence aggregation of sea cucumbers. They concluded that the conspecific attraction in sea cucumbers can also be related to favourable habitats with sufficient quality food. This study was conducted in aquaculture conditions with *Holothuria scabra*. More studies need to be done *in situ* conditions to evaluate whether this pheromone is found in other species and whether the pheromones involved in spawning are similar to those found in the study of Claereboudt *et al.* (2023). As the high densities and the stability characterizing patches of *H. leucospilota* in Reunion Island, the hypothesis of a stable environment with quality food can be proposed. Then conditions for conspecific attraction seem to be present. Such attraction should also be tested for *H. atra* patch.

In conclusion, no generalization of sedimentary factors driving sea cucumber distribution can be asserted and only local studies can solve this question. Anthropogenic disturbances seem to favour a high-density patchy distribution for *H. atra*, while the identification of environmental factors driving distribution of *H. leucospilota* and *S. chloronotus* need more investigation. Additional environmental factors could influence sea cucumbers distribution and should be monitored for a complete understanding, such as salinity (Jobson *et al.*, 2021), desiccation (Hou *et al.*, 2019), water velocity (Barkai, 1991), depth (Bulteel *et al.*, 1992), water temperature and habitat complexity (Bonham & Held, 1963) or other factors from water column, such as pH and nitrite concentration (Asha *et al.*, 2015). Physiological studies could be conducted to identify any substances related to conspecific attraction.

Multiseasonal monitoring highlights three distinct patterns of temporal dynamics of sea cucumber species

This study evidences that sedimentary factors are fluctuating at seasonal scale, with a mean of variation of 8% for total organic matter load, 150% for fine particles, 24% for chlorophyll *a*, 31% for $\Delta^{13}\text{C}$ and 33% for $\Delta^{15}\text{N}$. Surprisingly, despite these fluctuating concentrations, the three sea cucumber species monitored during this study exhibited three distinct patterns of temporal dynamics. Densities previously recorded for *H. leucospilota* (Conand & Mangion, 2002; Mangion, 2003; Bédier, 2013; Cuvillier, 2016; Pierrat, 2018) were lower than these found during this monitoring work, for all sites. Moreover, the density and length of *H. leucospilota* were stable over seasons between Winter 2019 and Winter 2021. Populations of *H. leucospilota* have therefore extended over years and, as a consequence this species has now high densities ($> 1 \text{ ind.m}^2$) at three sites, MNS, PLA and TE, whereas formerly it was only present in high density at MNS (Conand & Mangion, 2002) and PLA (P.F. observation personal). Purwati & Luong-van (2003) indicated that *H. leucospilota* mainly used asexual reproduction in Darwin waters (Australia), leading to high-density. Conversely, Conand, Morel & Mussard (1997) have studied the reproduction of *H. leucospilota* in Reunion Island by visual census, recording individuals resulting from transversal fission. They evaluated a low fission rate, about 5%, and concluded that asexual reproduction was rather low for

H. leucospilota populations. However, during this study no individual resulting from fission had been observed, meaning that the fission rate was close to zero. Meanwhile, genetic analyses at the same sites highlighted that individuals of *H. leucospilota* did not share any multi-locus genes (MLG) (Pierrat et al, 2023). These two-information combined with stable length and density lead to hypothesize that *H. leucospilota* may not use asexual reproduction anymore. In consequence, the main mode of reproduction of *H. leucospilota* populations at Reunion Island, is sexual reproduction through gamete spawning. The high-densities of *H. leucospilota* observed in Reunion Island reefs are therefore uncorrelated with the use of asexual reproduction. Sexual reproduction could then bring new individuals into the patches. As almost no juveniles could be found in the patches, patch stability can depend on immigration of adults living over the reef. Noteworthy, during the snorkelling sessions between sites, most of the numerous specimens observed outside of the high-density patches belonged to the *H. leucospilota* species (J.P and A.M., personal observations). This scenario strengthens the conspecific attraction hypothesis. Immigration of adults from outside of the high-density patches by conspecific attraction may have compensate the mortality and lead to such high densities.

Concerning the density of *H. atra*, a slight decrease in density has existed for two decades, with higher density reported in Jaquemet *et al.* (1999) than in Mangion (2003), but still above 4 ind.m⁻². This decrease seems stronger over years as density below 4 ind.m² with no clear seasonal pattern was assessed during this study. Only one patch of high-density of *H. atra* has been observed across the reefs of L'Hermitage/La Saline, and no individual was noticed in Étang-Salé reef. The fission rate of *H. atra* has also been studied in PLA site and has decreased drastically through years, with about 20% in 1995 (Conand, 1995), 15% in 2004 (Conand, 2004) and no individual underwent transversal fission during our monitoring, meaning that the fission rate seems to be currently reaching zero for this species also. Additionally, Pierrat *et al.* (2022b) evidenced that *H. atra* individuals at Reunion Island have their own MLG, and only a simulation with lower microsatellite markers has found few multi-locus lineages (i.e. MLGs close enough to be part of the same MLL) among *H. atra* populations. The decrease in density of *H. atra* is probably linked to the diminution of the use of asexual reproduction.

Finally, the temporal variability of the density of *S. chloronotus* seems to be the most alarming. Density of this species has drastically decreased on the three sites where high-density was previously recorded (Conand *et al.*, 1998; Burgos & Hollinger, 2007; Bédier, 2013). High-density is currently reported at TE only. Density at PAS and ES has decreased over the short temporal scale of this study, combined with individual length reduced by more than half at ES. As for *H. leucospilota* and *H. atra*, the fission rate of *S. chloronotus* has decreased from 24% (Conand *et al.*, 1998) to 11% (Bédier, 2013) and 0% (this study), with no pre or post-division individuals observed during our monitoring. Conversely to the two others species, genetic analyses have highlighted the use of asexual reproduction for *S. chloronotus* (Pirog *et al.*, 2017; Pierrat *et al.*, 2023), with individuals sharing MLG. General trend is a slight increase of the clonal richness from 0.09 in 2013/2014, 0.10 in 2015/2016 (Pirog *et al.*, 2017) to 0.24 (Pierrat *et al.*, 2023). The pattern observed at PAS, where the density decreased while the length increased, can be considered as another evidence of the decrease of asexual reproduction by *S. chloronotus*. Some observations illegal harvesting of sea cucumbers in Reunion Island reefs have been reported on social network, what might also contribute to the decrease of *S. chloronotus* populations, a species that has a medium retail price (Purcell *et al.*, 2018). According to Hernández-Flores *et al.* (2018), high-density patchy sea cucumber populations are likely to be over-harvested due to their high accessibility, and can be depleted and destabilized in a very short time. Other unstudied factors could also constrain populations, such as diseases (Wang *et al.*, 2007; Han, Keesing & Liu, 2016b), global climate change (Buccheri *et al.*, 2019), or predation (Kropp, 1982; Francour, 1997; Papafitsoros, 2023).

In conclusion, temporal long-term monitoring data are concomitant with the genetic data. The three species monitored during this study exhibited distinct patterns of temporal dynamics, with *H. leucospilota* populations having increased and now being stable, with *H. atra* with no particular pattern, and with *S. chloronotus* densities having drastically decreased. Only populations of *H. leucospilota* seem to thrive now within the reef of L'Hermitage/La Saline.

Feeding behaviour evidences generalist vs specialist species

The evaluation of feeding parameters brings useful information concerning the profiles of the species studied. The moving speeds and ingestion rates of *H. leucospilota* and *H. atra* did not show any variation during the day, therefore these results confirm that these two species have a continuous diurnal feeding rhythm, as reported previously (Klinger & Johnson, 1998; Mangion *et al.*, 2004). Conversely, *S. chloronotus* seems less active in the early morning (8 am), meaning that this species has a diurnal rhythm, which is congruent with other studies (Yamanouchi, 1956; Franklin, 1980; Klinger & Johnson, 1998). Yamanouchi (1956) showed that *S. chloronotus* hides under coral between 00 and 10 am for fasting, then goes on the sand for foraging on daylight. The speed of *S. chloronotus* was higher than these of two other *Holothuria* species. The speed of sea cucumbers is attributed to foraging activities, and more available the food is, less the sea cucumbers move (Klinger & Johnson, 1998; Uthicke & Karez, 1999; Conand, 2004). For instance, Mangion (2003) has estimated that mean speed of *H. leucospilota* and *H. atra* were significantly different between low-density site with poor sediments (120 and 170 cm.h⁻¹) and high-density site with enriched sediments (40 and 60 cm.h⁻¹). The ingestion rate of *S. chloronotus* was also higher than those of the two other *Holothuria* species, except at TE. If the ingestion rate is higher, it means that more sediments have to get through the gut of *S. chloronotus* to fulfil its nutrient requirements. The foraging depth of *S. chloronotus* tentacles is only one centimetre, whereas those *H. atra* can reach four centimetre depth (Taddei, 2006). Regarding all the arguments, diurnal pattern, moving speed, ingestion rate and depth of foraging, *S. chloronotus* seems to be a specialist species, whereas *H. leucospilota* and *H. atra* are generalist species, as already suggested by Uthicke (1999). As this study highlighted that the sediment parameters of Reunion Island reefs are fluctuating across seasons, the specialist feeding behaviour of *S. chloronotus* appears to be a disadvantage. An exhaustive exploration of the diet of these three species could confirm these results. The specialist behaviour of *S. chloronotus* can negatively influence the density, as more energy is allocated to foraging for fulfil daily nutrient requirements, compared to the two other species. The feeding behaviour of the species appears therefore to be a key factor explaining the distribution and density of sea cucumber in Reunion Island.

Conclusion

This study highlights some relations between sedimentary factors and patchy distribution of sea cucumbers from Reunion Island. Anthropogenic disturbances due to terrestrial waters inputs in reefs, may have contributed to the patchy distribution observed for *H. atra*, with higher total organic matter and $\Delta^{13}\text{C}$ contents. Patchy distribution of *H. leucospilota* is associated with the cover of seagrasses while no sedimentary factors can explain the patchy distribution of *S. chloronotus*. Multiseasonal monitoring evidences that the three species studied displayed three distinct patterns of temporal dynamics. Since the 1990s, the densities of *H. leucospilota* have increased and are now stable. Since populations of *H. leucospilota* stopped using asexual reproduction, conspecific attraction could maintain high-density patches of *H. leucospilota*. The temporal dynamics of *H. atra* presented no particular pattern. Finally, the densities of *S. chloronotus* have drastically decreased. This may be due to a synergy between the diminution of asexual reproduction and potential illegal over-harvesting, or other factors to be identified. The specialist behaviour demonstrated by *S. chloronotus* could be a limiting factor for the sustainability of this species in these reefs. Further studies should focus on other environmental factors in other reef compartments to fully explain to patchy distribution of sea cucumbers at Reunion Island. Physiology of wild sea cucumbers can be explored to find any markers for conspecific attraction. Spatio-temporal monitoring appears as a useful tool to understand trajectories of wild sea cucumber populations.

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Supplementary Material

Supplementary material 11: Pearson's correlation results of environmental variables

Variables clustered together for both gradient	r value	Df	P-value
<i>Red macroalga & Cyanobacteria</i>	0.421	243	< 0.001
<i>Erected dead coral & Coral rubble without turf</i>	0.494	243	< 0.001
<i>Sponges & Living coral without turf</i>	0.251	243	0.005
<i>Sponges & Living coral with turf</i>	0.361	243	< 0.001
<i>Living coral without turf & Living coral with turf</i>	0.299	243	0.001
Variables removed for back-reef gradient	r value	Df	P-value
<i>Organic matter load & Chlorophyll a</i>	0.393	37	< 0.001
<i>Organic matter load & $\Delta^{15}N$</i>	0.704	37	< 0.001
<i>Organic matter load & $\Delta^{13}C$</i>	0.664	37	< 0.001
<i>Chlorophyll a & $\Delta^{15}N$</i>	0.595	37	< 0.001
<i>Chlorophyll a & $\Delta^{13}C$</i>	0.597	37	< 0.001
<i>$\Delta^{15}N$ & $\Delta^{13}C$</i>	0.921	37	< 0.001
<i>Coral rubble with turf & Fines particles</i>	0.227	37	< 0.001
Variables removed for shore to ocean gradient	r value	Df	P-value
<i>Organic matter load & $\Delta^{15}N$</i>	0.316	19	0.011
<i>Dead coral & Chlorophyll a</i>	0.229	19	0.001

Supplementary material 12: Final model of each species (*H. leucospilota*, *H. atra* and *S. chloronotus*) for back-reef and ocean to shore gradient.

Back-reef gradient	Final Model	R²
<i>H. leucospilota</i>	= site + chlorophyll a + seagrasses + dead coral + living erected organisms + coral rubble with turf + chlorophyll a × site + coral rubble with turf × site + dead coral × site + living erected organisms × site	0.745
<i>S. chloronotus</i>	= site + season + chlorophyll a + dead coral + coral rubble with turf + chlorophyll a × season + coral rubble with turf × season + dead coral × season	0.494
Shore to ocean gradient	Final Model	R²
<i>H. atra</i>	= station + season + organic matter load + sand + dead coral + fine particles + coral rubble with turf + living erected organisms + algocyano + $\Delta^{13}C$ + $\Delta^{13}C$ × season + organic matter load × season	0.897
<i>H. leucospilota</i>	= station + season + sand + dead coral + fine particles + coral rubble with turf + $\Delta^{13}C$ + $\Delta^{13}C$ × station + fine particles × season	0.462

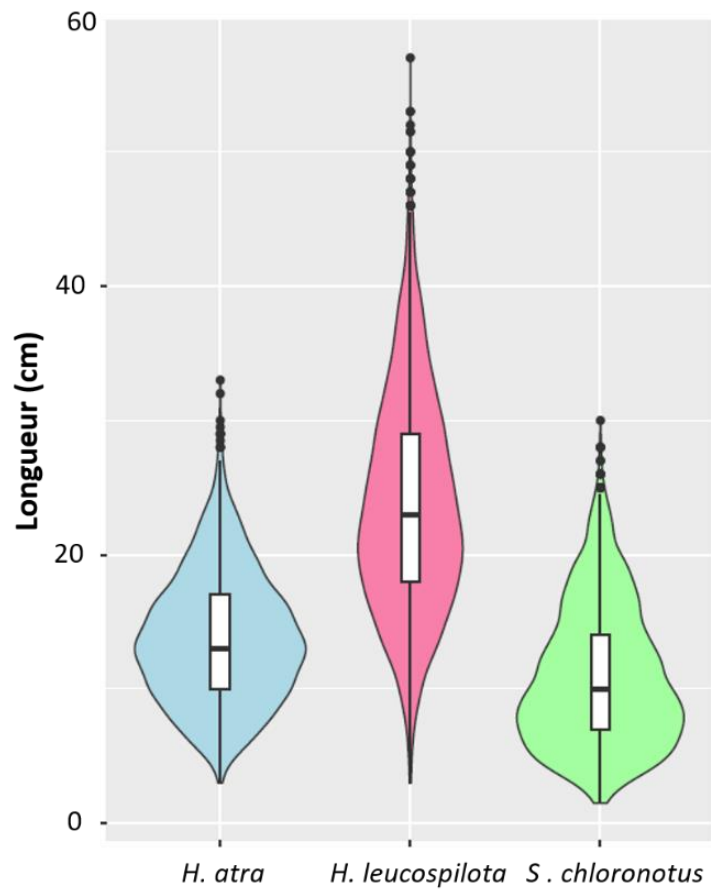
Supplementary material 13: Distribution of lengths of the three species monitored during this study.

Holothuria atra: in blue (N= 2012);

Holothuria leucospilota: in pink (N= 3876);

Stichopus chloronotus: in green (N= 2581)

Violin Plot (using ggplot2 package on R).



Supplementary material 14: Spatio-temporal variations of sedimentary factors from the back-reef depression gradient. Values are mean \pm se. Different letters indicate significant difference between seasons or sites (Pvalue < 0.005). Results of Tukey-HSD tests (except for $\Delta^{13}\text{C}$ seas seasonal variation where Student Newman-Keuls test was used to reveal the significant difference highlighted by ANOVA).

Sedimentary factors	Winter 2019	Summer 2020	Winter 2020	Summer 2021	MNS	PAS	TE	ES
Sand %	<i>a</i>	<i>ab</i>	<i>b</i>	<i>b</i>	<i>ac</i>	<i>b</i>	<i>ab</i>	<i>c</i>
	33.6 \pm 1.44	26.6 \pm 1.36	23.9 \pm 1.13	23.9 \pm 1.19	24.7 \pm 1.38	35.8 \pm 1.69	30.2 \pm 1.15	19.1 \pm 0.65
Coral rubble with turf %	<i>a</i>	<i>a</i>	<i>ab</i>	<i>b</i>	<i>a</i>	<i>ab</i>	<i>b</i>	<i>ab</i>
	31.1 \pm 2.73	29.6 \pm 3.88	38.4 \pm 2.40	47.4 \pm 2.32	41.3 \pm 2.74	39.6 \pm 3.54	29.4 \pm 3.29	36.3 \pm 2.06
Living erected organisms %	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
	16.7 \pm 2.63	18.1 \pm 3.33	17.6 \pm 2.79	18.3 \pm 3.40	0.59 \pm 0.19	7.42 \pm 0.83	17.8 \pm 2.10	40.8 \pm 2.12
Dead corals %	<i>ab</i>	<i>a</i>	<i>b</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>a</i>
	13.8 \pm 3.30	15.9 \pm 4.18	5.54 \pm 1.05	4.68 \pm 1.32	7.89 \pm 1.44	7.17 \pm 2.24	21.2 \pm 4.05	2.34 \pm 0.59
Algocyano %	<i>a</i>	<i>ab</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>
	0 \pm 0	3.65 \pm 1.85	8.56 \pm 2.05	1.29 \pm 0.45	2.87 \pm 0.87	9.60 \pm 2.83	1.54 \pm 1.06	1.48 \pm 0.72
Seagrasses %	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>b</i>	<i>b</i>
	4.85 \pm 1.71	6.26 \pm 2.4	5.82 \pm 1.67	4.47 \pm 1.49	22.6 \pm 1.68	0 \pm 0	0 \pm 0	0 \pm 0
Total organic matter load %	<i>a</i>	<i>ab</i>	<i>b</i>	<i>ab</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>c</i>
	2.40 \pm 0.04	2.30 \pm 0.06	2.23 \pm 0.03	2.35 \pm 0.05	2.27 \pm 0.03	2.38 \pm 0.02	2.61 \pm 0.02	2.01 \pm 0.03
Fine particles %	<i>ab</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
	0.90 \pm 0.05	1.10 \pm 0.05	0.81 \pm 0.07	2.04 \pm 0.07	1.05 \pm 0.08	1.31 \pm 0.13	0.99 \pm 0.10	1.25 \pm 0.07
Chlorophyll <i>a</i> mg.m²	<i>a</i>	<i>b</i>	<i>b</i>	<i>ab</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>b</i>
	107 \pm 5.75	133 \pm 7.62	131 \pm 5.86	129 \pm 6.86	134 \pm 5.61	128 \pm 6.07	143 \pm 4.86	93.5 \pm 6.75
$\Delta^{13}\text{C}$ ‰	<i>a</i>	<i>b</i>	<i>b</i>	<i>ab</i>	<i>a</i>	<i>bd</i>	<i>ad</i>	<i>c</i>
	-8.40 \pm 1.51	-6.43 \pm 1.70	-6.52 \pm 1.12	-7.45 \pm 1.29	-3.61 \pm 0.50	-0.86 \pm 0.12	-2.56 \pm 0.53	-20.4 \pm 0.70
$\Delta^{15}\text{N}$ ‰	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>b</i>	<i>c</i>
	2.22 \pm 0.65	2.95 \pm 0.66	2.53 \pm 0.61	2.80 \pm 0.54	2.52 \pm 0.44	5.48 \pm 0.14	5.60 \pm 0.20	-2.78 \pm 0.29

Supplementary material 15: Spatio-temporal variations of sedimentary factors from the shore-to-ocean gradient. Values are mean \pm se. Different letters indicate significant difference between seasons or sites (Pvalue < 0.005). Results of Tukey-HSD tests.

Sedimentary factors	Winter 2020	Summer 2021	Winter 2021	PLASHORE	PLAMID	PLAOCEAN
Sand %	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
	51.0 \pm 5.84	27.0 \pm 2.36	48.6 \pm 2.64	40.1 \pm 4.16	46.3 \pm 4.45	33.7 \pm 4.70
Coral rubble with turf %	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
	34.3 \pm 5.96	62.2 \pm 2.79	36.9 \pm 4.71	44.6 \pm 4.24	41.3 \pm 4.60	55.2 \pm 6.06
Living erected organisms %	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
	5.14 \pm 1.07	5.32 \pm 0.85	6.01 \pm 1.02	7.17 \pm 1.13	4.47 \pm 0.95	4.76 \pm 0.65
Dead corals %	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
	6.03 \pm 2.39	3.01 \pm 0.87	8.38 \pm 2.18	5.71 \pm 1.67	5.92 \pm 2.12	4.60 \pm 1.53
Algocyano %	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
	3.55 \pm 1.68	2.49 \pm 1.05	0.05 \pm 0.04	2.37 \pm 1.59	2.22 \pm 1.03	1.70 \pm 0.77
Total organic matter load %	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>b</i>
	2.47 \pm 0.05	2.62 \pm 0.03	2.43 \pm 0.02	2.62 \pm 0.05	2.49 \pm 0.03	2.46 \pm 0.02
Fine particles %	<i>a</i>	<i>b</i>	<i>c</i>	<i>a</i>	<i>a</i>	<i>a</i>
	0.73 \pm 0.12	1.92 \pm 0.10	0.29 \pm 0.06	1.23 \pm 0.14	1.46 \pm 0.14	1.50 \pm 0.15
Chlorophyll <i>a</i> mg.m²	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>	<i>a</i>
	109 \pm 4.55	100 \pm 6.79	94.6 \pm 10.4	112 \pm 7.90	99.4 \pm 8.49	92.2 \pm 5.56
$\Delta^{13}\text{C}$ ‰	<i>a</i>	<i>b</i>	<i>a</i>	<i>a</i>	<i>b</i>	<i>b</i>
	0.03 \pm 0.03	-0.19 \pm 0.02	0.13 \pm 0.05	0.09 \pm 0.05	-0.07 \pm 0.03	-0.12 \pm 0.03
$\Delta^{15}\text{N}$ ‰	<i>a</i>	<i>b</i>	<i>c</i>	<i>a</i>	<i>a</i>	<i>a</i>
	3.78 \pm 0.10	4.12 \pm 0.05	1.93 \pm 0.09	3.50 \pm 0.19	3.33 \pm 0.26	3.37 \pm 0.21

Partie II –

Alimentation et stress des holothuries dans un environnement pollué

Chapitre 4 –

Bactéries benthiques dans le régime alimentaire de trois espèces d'holothuries : processus d'ingestion, de sélection et d'assimilation



*Individu de Holothuria leucospilota
s'alimentant à l'aide de ses tentacules buccaux
à Trou d'Eau dans le récif de l'Hermitage-La Saline*

-
26.01.19

Résumé

La qualité et la quantité de ressources alimentaires disponibles dans un habitat est souvent en lien avec la distribution des espèces associées. La dynamique spatio-temporelle des ressources alimentaires est l'un des facteurs influençant la dynamique des populations marines. Pour comprendre cette dynamique, il faut déjà identifier les ressources impliquées dans le régime alimentaire des espèces.

Dans le cadre des holothuries, plus particulièrement des holothuries dépositives, cette identification peut être rendue difficile puisque la plupart des ressources ingérées sont concentrées dans le sédiment, comprenant des bactéries, des organismes photosynthétiques, la méiofaune, des minéraux et de la matière détritique. Bien que de nombreuses anciennes études ont essayé de comprendre le régime alimentaire de différentes espèces d'holothuries, un flou persiste dû à des limitations techniques. Aujourd'hui, le développement de nouveaux outils comme le métabarcoding permet d'obtenir une vision large des espèces impliquées dans le régime alimentaire des nombreuses espèces.

À La Réunion, le régime alimentaire des trois espèces d'holothuries présentes en forte densité, *Holothuria atra*, *Holothuria leucospilota* et *Stichopus chloronotus*, n'a pas été examiné.

Ce chapitre vise, dans un premier temps, à faire la synthèse des connaissances sur la nutrition des holothuries, incluant le comportement alimentaire, la morphologie et les processus digestifs, et ce qui est connu du régime alimentaire. Puis, dans un second temps, ce chapitre se concentre sur l'étude de la dynamique spatio-temporelle des bactéries benthiques à La Réunion et leurs implications dans les processus d'ingestion et d'assimilation chez les holothuries.

Les résultats de ce chapitre sont publiés dans *Biological Reviews* concernant la revue sur l'alimentation des bactéries et en préparation pour être publiés concernant le régime alimentaire bactérien.

Sophistication in a seemingly simple creature: a review of wild holothurian nutrition in marine ecosystems

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Abstract

Holothurians are marine invertebrates that are among the most widespread benthic megafauna communities by both biomass and abundance in shallow-water and deep-sea ecosystems, their functions supporting important ecological services worldwide. Despite their simple appearance as sea cucumbers, holothurians show a wide range of feeding practices. However, information on what and how these animals eat is scattered and potentially confusing. We provide a comprehensive review of holothurian nutrition in coastal and deep-sea ecosystems. First, we describe morphological aspects of holothurian feeding and the ultrastructure of tentacles. We discuss the two processes for food capture, concluding that mucus adhesion is likely the main method; two mucous cells, type-1 and type-2, possibly allow the adhesion and de-adhesion, respectively, of food particles. Secondly, this review aims to clarify behavioural aspects of holothurian suspension- and deposit-feeding. We discuss the daily feeding cycle, and selective feeding strategies. We conclude that there is selectivity for fine and organically rich particles, and that feeding through the cloaca is also a route for nutrient absorption. Third, we provide a wide description of the diet of holothurians, which can be split into two categories: living and non-living material. We suggest that Synallactida, Molpadida, Persiculida, Holothuriida and Elasipodida, ingest the same fractions, and emphasise the importance of bacteria in the diet of holothurians.

Key words: holothurians, tentacle ultrastructure, bud epidermis, mucous cell, trophic mode, selective feeding, dietary, proteobacteria, plastic particles.

I. Introduction

Dating back 460 million years, holothurians (commonly called sea cucumbers) are ubiquitous marine echinoderms belonging to the class Holothuroidea. An initial classification was established by Pearson (1914), who attempted to organise the species, under the name *Holothuria* L., into groups based on their gross morphology. Pawson & Fell (1965) subsequently proposed a classification based on tentacle, body, and calcareous ring morphology, defining five orders: Dendrochirotida, Apodida, Molpadida, Elasipodida, and Aspidochirotida. Until recently, this was the most widely used classification for holothurians. When the first broad-scale molecular phylogenetic analyses of Holothuroidea were completed, these deeply altered the previous classification through the suppression of the order Aspidochirotida, which was revealed to be polyphyletic. Species previously under Aspidochirotida have since been placed into three new orders: Synallactida, Persiculida (in part), and Holothuriida (Miller *et al.*, 2017). There are more than 1,752 accepted holothurian species (WoRMS, 2020), with new species being described each year.

Holothurians have a global distribution, colonising all biotopes of the ocean from the polar front (Lawrence & Guille, 1982; Féral & Magniez, 1985; Gutt, 1990; Post *et al.*, 2017; O’Loughlin, Bardsley & O’Hara, 2020) to the tropical zone (Sloan & von Bodungen, 1980; Wiedemeyer, 1994; Asha *et al.*, 2015; Resueño & Angara, 2020), with most species inhabiting the tropical Indo-West Pacific region (Conand, 1990). They have also colonised all depths, from shallow-water (Jaquemet, Rousset & Conand, 1999; Dissanayake & Stefansson, 2010; MacTavish *et al.*, 2012; Lee *et al.*, 2017) to hadal zones (Iken *et al.*, 2001; Jamieson *et al.*, 2011). They are among the most widespread benthic megafauna species in terms of biomass and abundance in many ecosystems, especially in the hadal zone, considered ‘the kingdom of Holothuroidea’ (Beliaev & Brueggeman, 1989; Kuhnz *et al.*, 2014), in coral reefs and lagoons (Uthicke, 1999; Wolfe & Davey, 2020), and in sheltered marine shallow habitats (Conde, Diaz & Sambrani, 1991).

Some species of holothurians are considered as luxury food (bêche-de-mer, trepang or hai-som), medicines, and aphrodisiacs in many Asian countries (Conand, 1990; Lovatelli *et al.*, 2004; Shiell & Uthicke, 2005; Toral-Granda, Lovatelli & Vasconcellos, 2008). More than 70 species of holothurians are commonly harvested (Purcell *et al.*, 2016), predominantly from the Indo-Pacific region (Kinch *et al.*, 2008; Conand, 2018). In some locations, populations of highly commercially valuable species have been decimated to a point that fishing regulations and regulatory measures alone may be insufficient to restore populations (Friedman *et al.*, 2011). This overexploitation is linked to a shift from traditional to semi-industrial fisheries (Conand, 2001). Countries of the Indian Ocean, West Pacific and Latin America have active fisheries (Conand, 2018), and South-East Asia is considered the main world market (Rahman & Yusoff, 2017). The world fishery of holothurians quadrupled between 1955 and 2012 to satisfy the increasing Asian market for 'bêche-de-mer' (Rahman, Yusoff & Arshad, 2015). Consequently, many countries in the Indo-Pacific have prioritised sea cucumber aquaculture in their development plans (Jimmy, Pickering & Hair, 2012). In addition, restocking, sea ranching, and sea farming have been described as potential alternatives to reduce pressure on wild holothurian populations and their worldwide overexploitation. Three species have been registered recently in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix II: *Holothuria (Microthele) fuscogilva* Cherbonnier, *Holothuria (Microthele) nobilis* (Selenka), and *Holothuria (Microthele) whitmaei* Bell, each with high commercial value but declining natural stocks (Di Simone, Horellou & Conand, 2019).

Due to their global distribution favouring many local studies, and their importance in Asian culture, holothurians have gained increasing importance in recent decades. A search on *Google Scholar* using the key words 'sea cucumber' for general publications and 'bêche-de-mer' for those focusing on fisheries and aquaculture, shows that the number of articles on both topics has increased considerably since the 1990s (**Figure 16**).

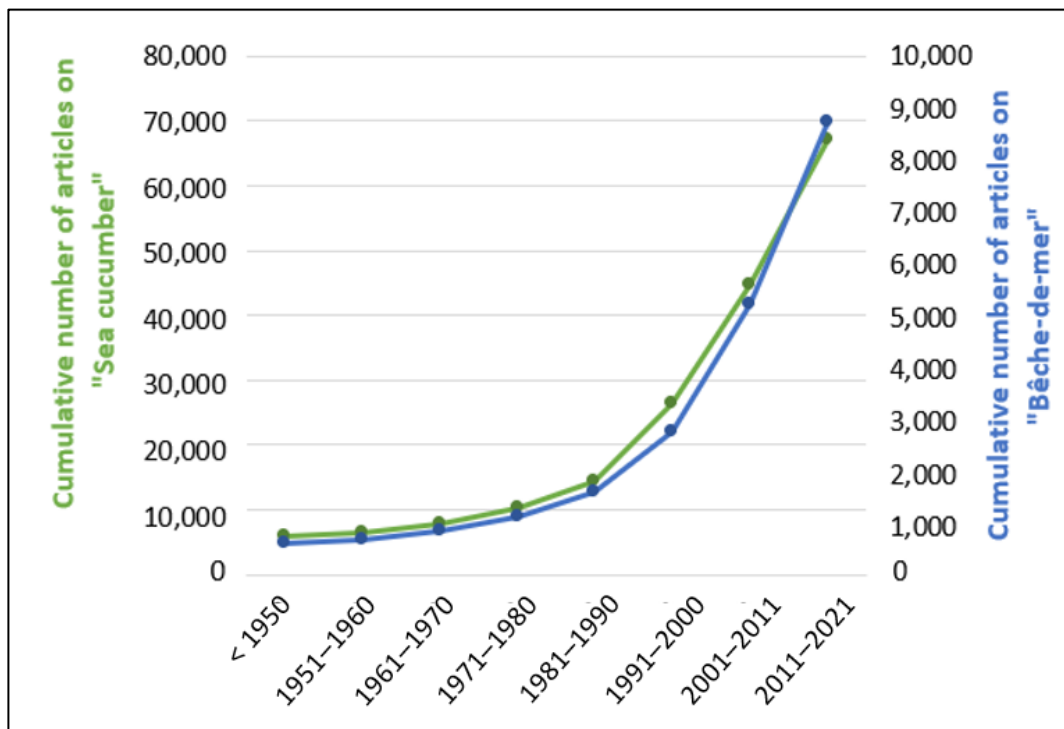


Figure 16: Cumulative number of articles on “sea cucumber” for general publications and “bêche-de-mer” for those focusing on fisheries and aquaculture since 1950. Note the different scales for the two axes.

The increasing literature provides scattered data about holothurian feeding, making it difficult to obtain a broad view on this topic. This review intends to assess our understanding of holothurian feeding. The nutritional aspects considered herein include feeding (the act of consuming food), digestion and absorption. To our knowledge, only one review has been published previously on the nutrition of holothurians (edited by Jangoux & Lawrence, 1982), about forty years ago. There is no comprehensive review covering the nutrition traits of holothurians in relation to their ecology, and collating information on their feeding patterns. While much information exists on the feeding of farmed holothurians, this relates only to controlled conditions. Thus, our aim herein is to analyse holistically the nutrition of wild holothurians.

II. Morphological and physiological aspects

(1) Tentacle structure and movements during ingestion

The number, size, and structure of tentacles varies among holothurian orders. Based on their structure, Massin (1982) defined five types of tentacles: dendritic, peltate, pinnate, digitate, and peltodendritic. The first four are common and broadly used in the description of tentacle morphology of holothurians and should be used as basic shapes. Species within Dendrochirotida possess dendritic tentacles whereas those of Molpadida possess digitate tentacles (Levin, 1989). Peltate tentacles, described as “cauliflower-like structures” (Bouland, Massin & Jangoux, 1982, p. 134) or “a nasturtium leaf with a central short stalk giving off horizontal branches” (Hyman, 1955, cf. Cameron & Fankboner, 1984, p. 193), are found within Elasipodida, Synallactida, Persiculida, and Holothuriida. In some cases, the tentacle structure shows variations among species within the same order, such as for Apodida species, which are found in coastal and deep-sea ecosystems and possess pinnate or digitate tentacles. Combinations of these four main types of tentacle can be found in the literature: (1) “peltodendritic tentacle” which is, according to Massin (1982), a combination of shaft of peltate tentacle and distal end of dendritic tentacle; (2) “peltatodigitate tentacle” which is a combination of shaft of peltate tentacle and distal end of digitate tentacle (Miller *et al.*, 2017). Peltodendritic tentacles are uncommon, whereas peltatodigitate tentacles are found in Apodida (Miller *et al.*, 2017).

Tentacle morphology may also differ between early and adult stages of the same species (Cameron & Fankboner, 1984). Generally, a tentacle possesses several shafts that end with one or more discs, composed of numerous apical papillae (**Figure 18A–B**). The apex of each papilla is characterised by the presence of buds (**Figure 18C**) with various cell types. The main difference in tentacle structure is the degree of branching of the main tentacle stalk (**Figure 17**), from an unbranched (digitate form), to a slightly branched (peltate form), to a highly branched (pinnate form) to an ultra-branched (dendritic form) structure. These differences reveal an adaptive radiation facilitating habitat and feeding specialisations (Sokolova, 1958; Hansen, 1975). From an ecological perspective, differences in tentacular

morphology among sympatric holothurian species with overlapping bathymetric ranges may allow resource partitioning to avoid competitive interactions (Roberts & Moore, 1997).

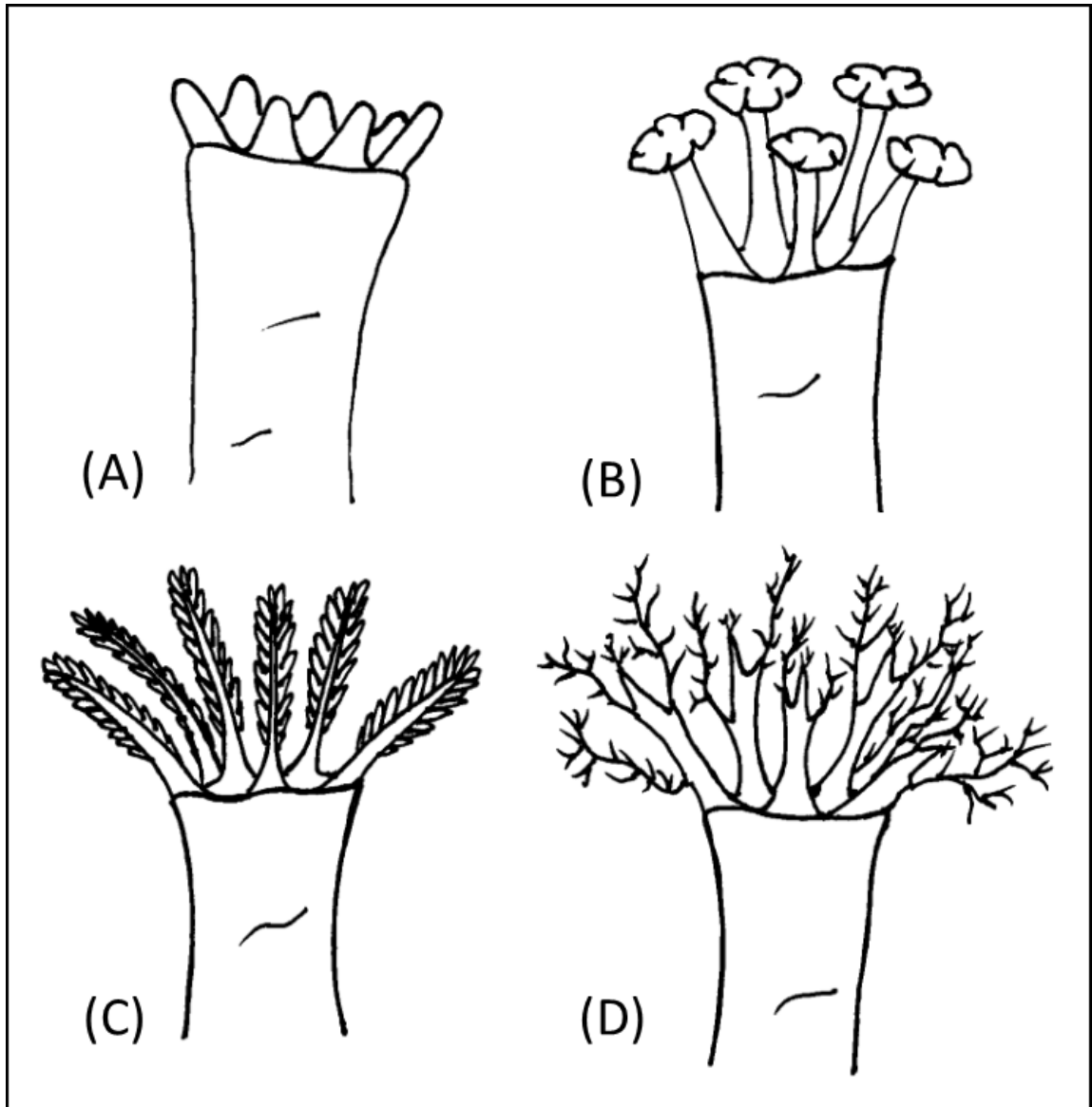


Figure 17: Examples of holothurian tentacle morphology. (A) Digitate (unbranched); (B) peltate (slightly branched); (C) pinnate (highly branched); (D) dendritic (ultra-branched).

A comprehensive review by Massin (1982) described the feeding mechanisms of deposit-feeding holothurians. When the animal initiates feeding, the tentacles expand into the water column, and are pressed onto or into the sediment surface where they perform investigatory movements (Bouland *et al.*, 1982). The tentacular movement is thought to be driven by the hydrostatic pressure of the ambulacral fluid of the water-vascular system and

the mesothelial muscles (Bouland *et al.*, 1982). Tentacles can also cooperate to capture large debris such as fragments of seagrass or macroalgae. In this case, when the desired food elements are located, the extremities of the tentacle retract first, thus entrapping the particles between the buds (Fankboner, 1978; Levin, 1989). Sokolova (1958) described how tentacle morphology can influence the collection of particulate food in deep-sea holothurian species; well-developed marginal processes on discs are capable of picking up single food particles from the sediment, whereas species with undifferentiated discs indiscriminately ingest the upper layer of the sediment. After collection, the tentacle continues to contract, bending towards the mouth and penetrating it. At this ingestion stage, mouth size is the limiting factor (Myers, 1977), although a proportion of captured particles is lost during transport to the mouth (Powell, 1977; Levin, 1989). As the tentacle penetrates the pharyngeal cavity, particle removal is facilitated by wiping the tentacle against the bulging pharynx wall (Cameron & Fankboner, 1984). Finally, the tentacle withdraws from the mouth and extends again to continue investigating for food sources.

(2) Food-capture mechanism

Understanding the mechanism(s) of capture of food particles in holothurians was approached *via* studies in functional morphology integrating data concerning the ultrastructure of the tentacles to determine the composition of the tentacle parts in contact with the ingested sediment. These studies, mainly carried out in the 1980s and 1990s, are unfortunately few in number. Sixteen species were investigated, mainly Dendrochirotida (13 species; Smith, 1983; McKenzie, 1987), two former Aspirochirotida (Bouland *et al.*, 1982; Cameron & Fankboner, 1984) and one Apodida (Flammang & Conand, 2004). Roberts & Moore (1997) and Fankboner (1981) used scanning electron microscopy to study the fine external structure of tentacles of four species of Elasipodida and five species of Dendrochirotida without detailing their cellular composition. Based on these studies and behavioural experiments, several authors have proposed mechanisms for the capture of food particles in several species (Roberts, 1979; Hammond, 1982; Bouland *et al.*, 1982; Cameron & Fankboner, 1984). Holothurians appear to use two methods for food collection: food particle ensnarement (a mechanical process) and food particle adhesion (a chemical process). Food

particle ensnarement was proposed in early studies about the functioning of the tentacles, since when adhesion has gained support as the principal mechanism involved in the capture of food particles.

(a) Food particle adhesion

Several authors described a mucus-like secretion on the oral tentacles and suggested a primary role in holothurian food capture (Bouland *et al.*, 1982). Roberts & Bryce (1982) described mucus-secretory cells in the tentacular epidermis of several tropical species and stated that the adhesive material would play a role in collecting food particles. Levin (1982), studying *Apostichopus japonicus* (Selenka), presumed that adhesion was a primary function of the tentacle during feeding. Similarly, Hammond (1982) observed mucus coating the tentacular surface and stated that adhesion is a significant factor in food collection for Synallactida, Persiculida, and Holothuriida, a statement reiterated by other authors working on temperate species (Smith, 1983; Costelloe & Keegan, 1984; Holtz & MacDonald, 2009).

Transmission electron microscopy studies have shown buds to be the sites of secretion. The bud epidermis can include six types of cells in the species investigated to date, with 3–5 of these cell types usually being present (**Table 10**). The functions of these cells have been deduced by drawing parallels with the roles of cells in the adhesive disc of podia of echinoderms. (1) ‘Support cells’ have been described in some Dendrochirotida (McKenzie, 1987) and in the only species of Apodida investigated (Flammang & Conand, 2004) (**Table 10**). (2) A ‘vesicular cell’ type has only been described in Apodida (Flammang & Conand, 2004) with unknown functions. (3) Ciliated cells, also named ‘unciliated cells’, ‘ciliated cells’ or ‘unciliated sensory cells’ have been observed in all species investigated (**Table 10**). Two roles have been suggested for this cell type: they could mechanically disengage particles (Fankboner, 1978) or they could be sensory (Flammang & Conand, 2004). Bouland *et al.* (1982) associated the bud structure of *Holothuria (Panningothuria) forskali* Delle Chiaje with Laverack’s (1974) description of chemosensory organs in marine invertebrates, where cilia are proposed to function as olfactory receptors, while microvilli are gustatory. Cilia are generally short and non-motile (Dorsett & Hyde, 1969; Schulte & Riehl, 1976), like those depicted by Bouland *et al.* (1982). The apical elements of the tentacles are the first structures to contact

substrates while the tentacles actively forage for food, strongly suggesting that the buds are likely to be chemosensory (Bouland *et al.*, 1982). The cells of the epidermis of each bud are adjacent to the epineural nervous plate, which is connected to the hyponeural nerve plexus of the tentacles (**Figure 18E–F**). Mesothelial muscles are present in each buccal tentacle (Fig. 3E; Bouland *et al.*, 1982; McKenzie, 1987). (4) ‘Mucous cells’, also called ‘mucocytes’ were observed in the tentacles of Dendrochirotida, Holothuriida and Apodida (**Table 10**). As similar cells are also observed outside the tentacles, their mucus-secreting role is probably to ensure the presence of a physical barrier on the epidermis. (5) Type-1 and (6) type-2 secretory cells are thought to play a direct role in the capture of food particles. Type-1 secretory cells, also referred to as ‘granular cells’, ‘glandular vesicular cells’ or ‘papillate cells’ (**Table 10**) are characterised by numerous dense-cored vesicles of 200–700 nm, with their diameter varying among species. These vesicles are found in many microvilli and are thought to be secreted into the cuticle. They may be homologues of the adhesive cells found in the podia of echinoderms. Type-2 secretory cells, also named ‘type-2 neurosecretory cells’ and ‘granular cells’, have been observed in Dendrochirotida and Apodida (**Table 10**). This cell type possesses numerous dense-cored vesicles of 60–130 nm, again with their diameter varying among species. These vesicles are found in the cell apex and also are thought to be secreted into the cuticle. They are thought to be homologues of de-adhesive cells found in the podia of echinoderms. Thus, the chemical capture of food particles could be performed by the secretion of type-1 secretory cells and, when the tentacles are placed in the oral cavity, adhesion could be removed by the secretion of type-2 secretory cells. This hypothesis is currently only theoretical and future functional morphology studies are needed, together with transcriptomic data to characterise the proteins expressed at the tentacle buds.

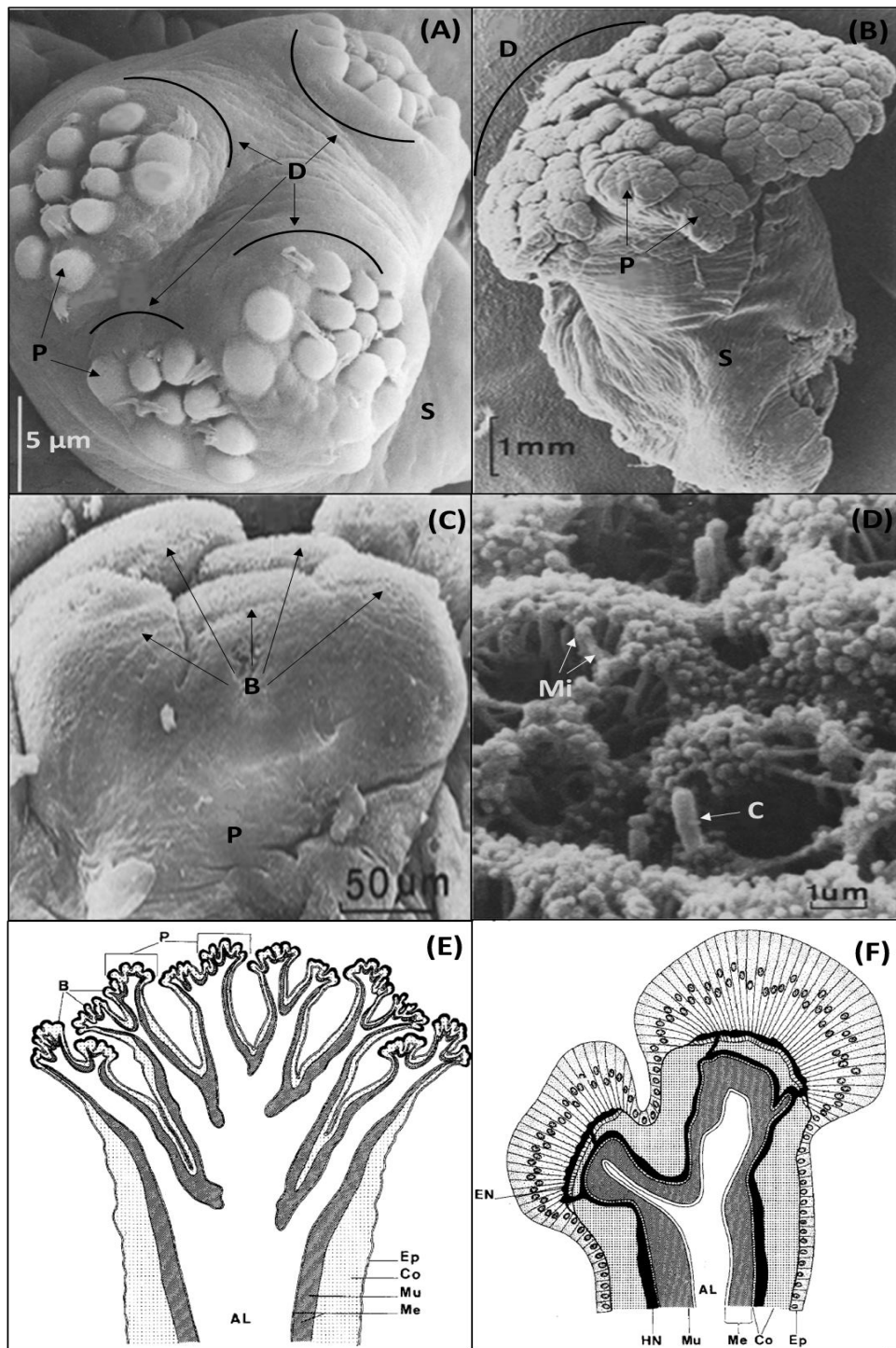


Figure 18: Fine ultrastructure of the holothurian tentacle. Digitate tentacle of (A) *Thyonidium* sp. and (B) *Holothuria forskali*. D, discs; P, Papillae; S, shaft. (C) Profile view of a five-bud papilla. B, buds; (D) Arrangement of cilia and microvilli on bud surface. C, cilium; Mi, microvilli. (E, F) Diagrammatic sections through a whole tentacle (E) and through a two-bud papilla (F). AL, ambulacral lumen; Co, connective tissue layer; EN, epineural nerve plexus; Ep, epidermis; HN, hyponeural nerve plexus; Me, mesothelium; Mu, muscles. Modified from Bouland et al. (1982) and McKenzie (1987).

Table 10: Ultrastructural composition of the tentacle buds of holothuroids with the nomenclatural terms used in analyses by various authors. The first column describes the corresponding cells observed in podia of echinoderms where a duo glandular adhesive system (adhesion and de-adhesion) has been identified.

Authors	Fankboner (1978)	Bouland <i>et al.</i> (1982)	Smith (1983)	Cameron & Fankboner (1984)	McKenzie (1987)	Flammang & Conand (2004)
Analysed taxon	Dendrochirotida	Holothuriida	Dendrochirotida	Synallactida	Dendrochirotida	Apodida
Number of species studied	1	1	1	1	11	1
Cell with no particular development of intracellular component	–	–	–	–	Support cell	Support cell (T-shaped cell) with 400–600 nm vesicles
Cell with large vesicle containing one spherule	–	–	–	–	–	Vesicular cell with numerous 4 µm vesicles
Cell with small apical cilia [presumed to be sensory cell, or cilia suggested to disengage food particles by Fankboner (1978)]	Uniciliated cell	Uniciliated cell	Ciliated cell	Uniciliated cell	Uniciliated cell	Uniciliated sensory cell
Cell filled with large clear vesicle (presumed to participate to the protection of the external surface)	Mucous cell	Mucous cell	–	–	Mucous cell (two different types)	Mucocyte (goblet-shaped cell) with 2 µm dense-cored vesicles
Cell with large dense-cored vesicles secreted into the cuticle (presumed to be similar to the adhesive cells identified in podia of echinoderms)	Papillary cell with 500 nm dense-cored vesicles released into the cuticle	Glandular vesicular cell	Papillate cell with 300–600 nm dense-cored vesicles released into the cuticle	Granular cell with 600–700 nm dense-cored vesicles released into the cuticle	Type-1 secretory cell with 200–400 nm dense-cored vesicles released into the cuticle	Type-1 secretory cell (spherical) with 250 nm dense-cored vesicles
Cell with small dense-cored vesicles (presumed to be similar to the de-adhesive cells identified in podia of echinoderms)	–	–	Granular cell with 130 nm dense-cored vesicles	–	Type-2 Presumed neurosecretory cell (due to low numbers) with 60–100 nm dense-cored vesicles	Type-2 Secretory cell (spherical) with 90 nm dense-cored vesicles

(b) Food particle ensnarement

Authors have long debated the mechanisms of food capture by which particles are trapped within bud interstices. Roberts (1979) suggested that expansion of the peltate tentacles caused inter-bud spaces to open on the tentacles, which could then mechanically trap particles when the tentacles retract. Cameron & Fankboner (1984) reported mechanical ensnarement to be of relatively little importance for *Parastichopus californicus* [now *Apostichopus californicus* (Stimpson)] since there is no reverse process of tentacle expansion/relaxation while the tentacle is in the pharyngeal cavity. Food detection is likely to be related to the ciliated cells as described in Section II.2, as the tentacles spread onto the substratum or in the water column.

(3) Digestive and assimilation processes in holothurians

(a) Digestive tract morphology

The nomenclature used for parts of the digestive tract varies among authors. Trefz (1958) distinguished between the mouth, pharynx, oesophagus, foregut, midgut, hindgut, cloaca and cloacal opening when studying the physiology of *Holothuria (Halodeima) atra* (Jaeger) (**Figure 19A**). Massin (1978), in his study on holothurian nutrition, focused mainly on *Holothuria (Holothuria) tubulosa* Gmelin (**Figure 19B**), differentiating the pharyngeal bulb surrounded by the calcareous ring, followed by the three portions of the digestive tract (foregut, midgut and hindgut), and finally the cloaca. Dividing the digestive tract between mouth and cloacal opening into three sections – foregut, midgut and hindgut – gives a broader view, and many studies describing holothurian feeding use this partitioning (e.g. Trefz, 1958; Ward-Rainey, Rainey & Stackebrandt, 1996; Taddéi, 2006; Plotieau, 2012; Amaro *et al.*, 2012).

The foregut includes the pharyngeal bulb, oesophagus, and the first descending loop of the intestine. The oesophagus is divided into two parts of equivalent lengths, both ending in a sphincter (sphincters 1 and 2) (**Figure 19B**). The midgut begins immediately beyond the first intestinal loop where the proximal tubules of the *rete mirabile* are attached and consists of the ascending anterior loop of the intestine. A third sphincter separates the midgut from

the hindgut. The hindgut, which represents approximately 70% of the total length of the gut, consists of the final posterior descending loop of the intestine and the cloaca from which respiratory trees emanate (Dolmatov & Ginanova, 2009). Only species from Pneumonophora have respiratory trees (Dendrochirotida, Holothuriida, Molpadida, Persiculida and Synallactida; Miller *et al.*, 2017). The descending loop of the foregut is paralleled by the dorsal haemal plexus, which extends posteriorly to the anal end of the animal where it loops back. The dorsal haemal plexus is a complex of pulsating channels attached to the dorsal midline of the foregut. Longitudinally along the ventral side of this region runs the ventral sinus. The *rete mirabile* is a complex of tiny tubules, entangled within the respiratory tree, and attached to the ascending loop of the midgut. These tubules terminate into the main channel of the dorsal haemal plexus.

Although the foregut, midgut, and hindgut have three different functions (accumulation, digestion, and assimilation), most species of Synallactida, Persiculida, and Holothuriida are able to assimilate food from these three parts (Massin, 1978).

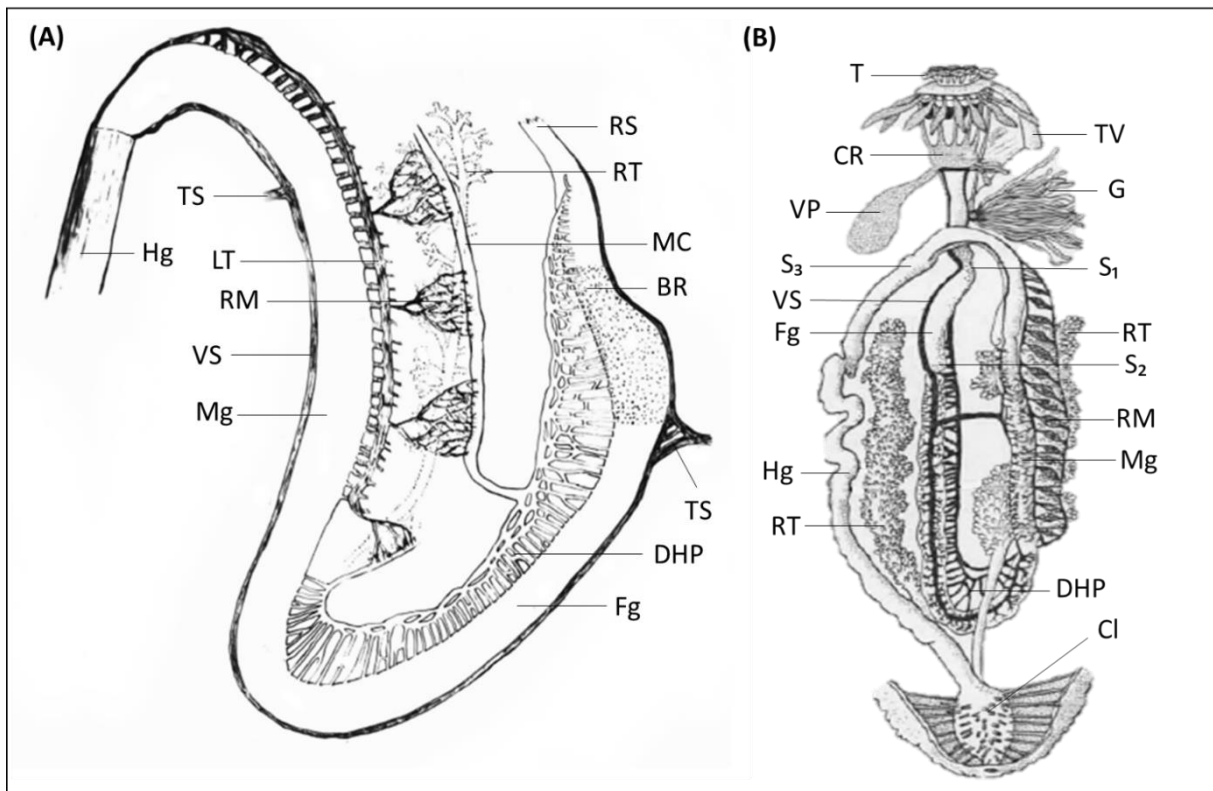


Figure 19: Digestive tract morphology of (A) *Holothuria atra* and (B) *H. tubulosa*. BR, brown region of the anterior foregut; Cl, cloaca; CR, calcareous ring; DHP, dorsal haemal plexus; Fg, foregut; G, gonads; Hg, hindgut; LT, longitudinal tube of rete mirabile; MC, main channel of dorsal haemal plexus; Mg, midgut; RM: rete mirabile; RS, region of severance; RT, respiratory tree; S₁, sphincter 1; S₂, sphincter 2; S₃, sphincter 3; T, tentacles; TS, transverse sinuses; TV, tentacle vesicles; VP, vesicle of Poli; VS, ventral sinus. Modified from Trefz (1958) and Massin (1978, inspired by Cuenot, 1948).

(b) Cell and enzymatic activities

The epithelia of the pharynx, oesophagus, and cloaca all contain T-shaped cells that are involved in the uptake of dissolved organic matter. The gut epithelium is composed of enterocytes with a filamentous coat for intracellular digestion (Féral & Massin, 1982). A broad range of hydrolytic gut enzymes has evolved in association with the diet (Féral, 1989). Hydrolytic enzyme activity has been reported within the digestive tract, on cell membranes (Féral, 1989), and inside cells (Lawrence, 1982). Roberts *et al.* (2000) reviewed the enzymes found in the gut of deposit-feeding holothurians (shallow-water species in Holothuriida, Synallactida, Apodida and Dendrochirotida and deep-sea species of Holothuriida, Persiculida,

Synallactida and Elasipodida), finding broad similarities in their hydrolytic enzymes. Gut enzymes include: (1) esterases that hydrolyse short-chain fatty acids, with strong activity throughout the gut (Fish, 1967; Féral, 1989), (2) lipases that hydrolyse long-chain fatty acids, with lowest activity (Clifford *et al.*, 1982; Féral, 1989; Manship, 1995), (3) proteases, (4) peptidases, which hydrolyse peptide bonds and may be important in the initial stages of protein digestion (Massin, 1984; Féral, 1989; Roberts *et al.*, 2000), (5) saccharidases, and (6) phosphatases, present at high concentrations in the gut tissue (Féral, 1989; Boetius & Felbeck, 1995).

Trefz (1958) observed phagocytic activity of abundant cells in the holothurian gut, such as round phagocytic amoebocyte cells that ingest and digest bacteria (*Bacillus subtilis*), but not indigestible particles.

(c) Obtaining nutrients through cloacal water retention

The major functions of cloacal ventilation are commonly described as respiration, excretion, and salt balance. Holothurians with respiratory trees (absent in Apodida and Elasipodida) have the ability to pump large volumes of water into and out of the cloacal opening. A similar pumping mechanism is reported for echinoderms such as holothuroids (Newell & Courtney, 1965; Brown & Shick, 1979) and edrioasteroids (Bell, 1977), annelids (Wolcott, 1981), and crustaceans (Fox, 1952). Innovative work, using radiography and stable isotope enrichment, demonstrated that the epithelium of the respiratory tree can assimilate dissolved organic matter such as monosaccharides and amino acids from the water column during cloacal water retention (Fontaine & Chia, 1968; Brothers, Lee & Nestler, 2015). The respiratory tree of *Cucumaria lactea* [now *Ocnus lacteus* (Forbes & Goodsir)] can assimilate dissolved ³H-labelled glycine and ³H-labelled glucose (Fontaine & Chia, 1968). The respiratory tree of *A. californicus* assimilated ¹⁵N-labelled amino acids and peptides (Brothers *et al.*, 2015) and ¹⁴C-labelled unicellular algae, suggesting the transfer of nutrients from the respiratory tree into the haemal system (Jaekle & Strathmann, 2013). Thus, it is possible that holothurians can obtain nutrients through their respiratory trees as a result of cloacal pumping, but additional studies are needed to clarify its importance to these animals

III. HOLOTHURIAN FEEDING BEHAVIOUR

As for all organisms, the movements and behaviour of holothurians will be driven by physiological requirements such as feeding, reproduction, or avoidance of stress factors (Mercier, Battaglene & Hamel, 1999; Pitt & Duy, 2004; Meng *et al.*, 2011, Hamel *et al.* 2019). A possible role of pheromones in intra- or inter-specific control of reproduction was recently hypothesised for holothurians (Marquet *et al.*, 2018).

(1) Deposit- or suspension-feeding

The first larval stage in holothurians, the auricularia, feeds on phytoplankton (planktotrophic) by means of cilia located on the epidermis. The second larval stage, the doliolaria, is non-feeding, and the final stage, the pentactula, develops the tentacles that are present in juveniles and adults. The first and second stages can be absent in holothurians with direct development (McEdward & Miner, 2001; Raff & Byrne, 2006). In general, juveniles and adults are particle-feeders, which usually exhibit either suspension- (in Dendrochirotida) or deposit-feeding (most other taxa) behaviour (Roberts *et al.*, 2000; Pawson, 1970; Bakus, 1973). Suspension-feeding holothurians tend to live in high-energy environments to allow them to intercept food particles (Taghon & Jumars, 1984). Deposit-feeding behaviour can be subdivided into three groups related to feeding depth (Roberts *et al.*, 2000). (1) Epibenthic deposit-feeders feed at the interface between the water column and sediment, or on the surface of seagrasses (Cuvillier, 2016), the sand scattered over the tegument of other specimens (e.g. *H. atra*; JP personal observations), boulders and corals (Roberts *et al.*, 2000; JP personal observations), or sponges (Hammond & Wilkinson, 1985). (2) Subsurface deposit-feeders feed below the sediment surface. (3) Funnel deposit-feeders create funnel-shaped depressions in sediments and feed on particles trapped in these funnels (Jumars, 1993).

Feeding mode may vary at the individual or population scale. According to Cadée (1984), many benthic individuals are opportunistic in their feeding mode. Some individuals of species in Dendrochirotida (Roberts *et al.*, 2000), Synallactida, Persiculida, and Holothuriida (Da Silva, Cameron & Fankboner, 1986) can shift between deposit-feeding and suspension-feeding. This may be possible if the individual is positioned in a negative geotropic orientation

(Da Silva *et al.*, 1986). *Leptopentacta elongata* (Düben & Koren) and other shallow-water species (Fankboner, 1981; Levin, 1989), are known to shift opportunistically from suspension- to deposit-feeding when the concentration of suspended particulate organic matter is low. This opportunistic behaviour is driven by environmental factors such as food pulses in shallow-water (tidal or seasonal fluctuations) (Cushing, 1959) and deep-sea ecosystems (Jumars, Self & Nowell, 1982; Billett *et al.*, 1983).

At the population scale, the feeding modes of some holothurian species can vary depending on their geographic location. *Holothuria (Thymiosycia) arenicola* Semper is an epibenthic feeder, feeding on surface layers of sand under coral debris in the Indo-West Pacific region and Cuba (Levin, 1989), whereas in the Bahamas, it feeds on particles in sediments (Mosher, 1980) as a subsurface deposit-feeder. The underlying reasons for these intraspecific differences at a population scale, and their drivers, are poorly studied.

(2) Daily burrowing cycle

A study on the burrowing behaviour of shallow-water holothurians in Palao Island classified holothurians into two groups according to their feeding habits (Yamanouchi, 1939): (1) species that do not burrow into sediments or other substrates and feed continuously, such as *Holothuria (Halodeima) edulis* Lesson, *H. (Semperothuria) flavomaculata* Semper or *H. atra* (Yamanouchi, 1939; Trefz, 1958; Uthicke, 1994) and (2) species that show a daily cycle of burrowing and feeding (Yamanouchi, 1939, 1956; Mercier *et al.*, 1999; Lavitra *et al.*, 2009). The factors regulating burrowing and feeding cycles appear to be complex (Yamanouchi, 1939, 1956), with differences among species increasing the complexity of cues that drive such behaviour and impeding generalisation.

According to Yamanouchi (1956), most adults prefer to feed during the day, particularly in the early afternoon when the water temperature is highest (Mercier *et al.*, 1999) and productivity of the marine ecosystems is maximal (Heil *et al.*, 2004). Some species, such as *Stichopus chloronotus* Brandt, move under corals from 00:00 to 10:00 and then return to sediments to feed during the day (Yamanouchi, 1956). Similarly, *Stichopus variegatus* (now *Stichopus herrmanni* Semper) hides under seagrasses between 20:00 and 10:00 (Yamanouchi,

1956). Coulon & Jangoux (1993) argued that it may be more energetically advantageous for juveniles of *H. tubulosa* to stop feeding during the coldest hours of the day. Individuals exposed to abnormal water temperatures exhibit unusual behaviour and feeding activity (Kato & Hirata, 1990). The temperate species *A. japonicus* aestivates during the warmest months when water temperatures are between 20 and 24.5 °C (Choe, 1963), especially large and mature individuals (Liu *et al.*, 1996). Aestivating individuals hide under structures and enter a state of dormancy (Liu *et al.*, 1996; Yang *et al.*, 2005) in which feeding activity ceases (Yuan *et al.*, 2007) until water temperature becomes more favourable. During aestivation, *A. japonicus* individuals undergo a series of physiological and morphological changes (Wang *et al.*, 2008), losing 30–50% of their body mass (Liu *et al.*, 1996). The digestive tract degenerates to half its pre-aestivation size (Li *et al.*, 1996; Liu *et al.*, 1996). Physiological responses associated with digestion are reduced during aestivation, with lower activity of digestive enzymes in the principal portions of the gut (Cui, Dong & Lu, 2000). Similarly, under high temperatures (austral summer), *Holothuria (Metriatyla) scabra* Jaeger, changes its burrowing behaviour, remaining on the sediment surface and feeding frequently, whereas at low water temperatures (austral winter) it remains beneath the sediment surface for most of the day (Mercier, Battaglione & Hamel, 2000). Thus, seasonal patterns will play a major role in the regulation of the daily burrowing cycle of this species.

The effects of salinity on the foraging activities of holothurians are unclear. Mercier *et al.* (1999, 2000) found that *H. scabra* cease feeding when water salinity is the lowest, and burrow into the substrate. James & James (1994) reported conflicting results, with juveniles emerging during low tide. Skewes *et al.* (2006) reported that only a third of *H. scabra* adults emerge during high tide.

Some studies show conflicting results concerning daily burrowing cycles of juveniles and adults. On one hand, daily burrowing cycles can be similar for juveniles and adults of the same species. In Palao Island, *H. scabra* adults were observed burrowing in the sediment between 03:30 and 15:00 and feeding between 15:00 and 03:30 (Yamanouchi, 1956). In the Philippines, *H. scabra* juveniles burrow in sediments between 03:00 and 09:00 and remain hidden until they emerge to feed between 15:00 to 03:00 (Altamirano, Recente & Rodriguez,

2017). Despite their different life stages and different geographical regions, both species therefore feed during the same time periods. On the other hand, holothurian size has been reported to affect burrowing and daily cycles. Mercier *et al.* (1999) found the burrowing cycle of *H. scabra* to be driven by light for small juveniles (10–40 mm), which burrow at sunrise and emerge close to sunset, whereas temperature was more influential for intermediate-sized juveniles (40–140 mm), which emerge earlier in the afternoon. Like many echinoderms, the nocturnal feeding and movement habits of smaller holothurians might be an adaptation to avoid predation (Nelson & Vance, 1979; Hammond, 1982).

(3) Selectivity by deposit-feeding holothurians

Feeding selectivity is observed in many marine species of different taxa, such as Polychaeta (Petch, 1986; Shimeta, 1996), Gastropoda (Whitlatch & Obrebski, 1980), or Bivalvia (Hylleberg & Gallucci, 1975). Some other echinoderms show selectivity in feeding, including species in Asteroidea (Mellin *et al.*, 2017) and Echinoidea (Larson, Vadas & Keser, 1980; Boon & Duineveld, 2012).

To determine particle selectivity in deposit-feeding holothuroids, most studies compare the biochemical composition and concentration of compounds present within sediments around and beneath foraging individuals and in their foregut (Moriarty, 1982; Hammond, 1983; Amaro *et al.*, 2010). Examining particle selectivity is subject to the difficulty of investigating sediment ingestion at the appropriate scale (Lopez & Levinton, 1987). Particle selection is generally based on size, surface texture, specific gravity, and the presence of an organic coating (Taghon, 1989), but it can also be explained by mechanical processes (Jumars *et al.*, 1982). Discussion of particle selectivity by deposit-feeding holothurians usually refers to two different modes: the selection of particles with a specific grain size or selection of those with higher organic content. We found 29 studies describing both aspects of particle selectivity for shallow-water (23 studies) and deep-sea (six studies) holothurian species (**Table 11**). A total of 50 species have been studied, with only one study focused on suspension-feeding species (Hamel & Mercier, 1998).

Table 11: Synthesis of studies on selective feeding strategies in shallow-water and deep-sea holothurians.

Taxon	Size particle selectivity	Organic matter selectivity	Location	Authors
<i>Coastal species</i>				
> Apodida				
<i>Euapta lappa</i>	No	Not studied	Discovery Bay, Jamaica	Hammond (1982)
<i>Leptosynapta tenuis</i>	No	Not studied	North Carolina, USA	Powell (1977)
> Holothuriida				
<i>Actinopyga agassizi</i>	No	Not studied	Discovery Bay, Jamaica	Hammond (1982)
<i>Bohadschia bivittata</i>	Yes, 400 µm	Not studied	Pari Island, Indonesia	Roberts (1979)
<i>Bohadschia vitiensis</i>	Yes, gravel and coarse particles (spawning period: fine particles)	Yes	Hurghada, Egypt	Dar & Ahmad (2006)
<i>Holothuria arenicola</i>	No	Not studied	Discovery Bay, Jamaica	Hammond (1982)
	Not studied	No	Great Palm Island, Australia	Uthicke & Karez (1999)
	Yes, coarser particles	Yes	Red Sea coast, Egypt	Dar (2004)
	Yes	Not studied	Waikiki branch, Hawai	Trefz (1958)
<i>Holothuria atra</i>	Yes, gravel and coarse particles (in spawning period fine particles)	Yes	Hurghada, Red Sea, Egypt	Dar & Ahmad (2006)
	Not studied	Yes	Great Barrier Reef, Australia	Moriarty (1982)
	Yes, coarser particles	Yes	El Qasr reef, Saudi Arabia	Behairy, Beltagi & Rao (1985)
	Yes, 350 µm	Not studied	Pari Island, Indonesia	Roberts (1979)
<i>Holothuria cinerascens</i>	Yes, <63–500 µm	Not studied	Beacon Island, Australia	Roberts & Bryce (1982)
	Not studied	No	Great Palm Island, Australia	Uthicke & Karez (1999)
<i>Holothuria edulis</i>	Yes, 63–125 µm	Not studied	Beacon Island, Australia	Roberts & Bryce (1982)

<i>Holothuria forskali</i>	Yes, 60–200 µm	Yes	Algiers and Bou-Ismaïl Bay, Algeria	Mezali & Soualili (2013)
	Not studied	Yes	Toulon, France	Massin & Jangoux (1976)
<i>Holothuria grisea</i>	No	Not studied	Discovery Bay, Jamaica	Hammond (1982)
<i>Holothuria hartmeyeri</i>	Yes, 2000–3500 µm	Not studied	Beacon Island, Australia	Roberts & Bryce (1982)
<i>Holothuria hawaiiensis</i>	Yes, gravel and coarse particles (in spawning period fine particles)	Yes	Hurghada, Egypt	Dar & Ahmad (2006)
<i>Holothuria impatiens</i>	Yes, <63–500 µm	Not studied	Beacon Island, Australia	Roberts & Bryce (1982)
<i>Holothuria leucospilota</i>	Yes, coarse particles	Yes	Red Sea coast, Egypt	Dar (2004)
<i>Holothuria marmorata</i>	Yes, coarse particles	Yes	Red Sea coast, Egypt	Dar (2004)
<i>Holothuria mexicana</i>	No	Not studied	Discovery Bay, Jamaica	Hammond (1982)
<i>Holothuria nobilis</i>	Not studied	No	Great Palm Island, Australia	Uthicke & Karez (1999)
	Yes, 2000–3500 µm	Not studied	Beacon Island, Australia	Roberts & Bryce (1982)
<i>Holothuria cf. pervicax</i>	Yes, 2000–3500 µm	Not studied	Beacon Island, Australia	Roberts & Bryce (1982)
<i>Holothuria poli</i>	Yes, 200–600 µm	Yes	Algiers and Bou-Ismaïl Bay, Algeria	Mezali & Soualili (2013)
	No	Yes	Toulon, France	Massin & Jangoux (1976)
<i>Holothuria sanctori</i>	Yes, 60–200 µm	Yes	Algiers and Bou-Ismaïl Bay, Algeria	Mezali & Soualili (2013)
	Not studied	Yes	Canary Islands, Spain	Navarro <i>et al.</i> (2013)
<i>Holothuria scabra</i>	Yes, 125–250 µm	Not studied	Palk Bay, India	Baskar (1994)
<i>Holothuria stellati</i>	Yes, 60–200 µm	No	Algiers and Bou-Ismaïl Bay, Algeria	Mezali & Soualili (2013)
<i>Holothuria tubulosa</i>	Yes, 200–600 µm	Yes	Algiers and Bou-Ismaïl Bay, Algeria	Mezali & Soualili (2013)
	Not studied	Yes	Gulf of Naples, Italy	Amon & Herndl (1991)
	No	Yes	Toulon, France	Massin & Jangoux (1976)
> Molpadida				
<i>Molpadia oolitica</i>	Yes, smallest particles	Not studied	Cape Cod Bay, USA	Rhoads & Young (1971)

> **Synallactida**

<i>Australostichopus mollis</i>	No	Yes	Mahurangi Harbour, New Zealand	Slater <i>et al.</i> (2011)
<i>Isostichopus badionotus</i>	No	Yes	Bermuda	Sloan & von Bodungen (1980)
	No	Not studied	Discovery Bay, Jamaica	Hammond (1982)
<i>Parastichopus californicus</i>	Not studied	Yes	British Columbia, Canada	Paltzat <i>et al.</i> (2008)
<i>Parastichopus parvimensis</i>	No	Yes	Santa Catalina Island, USA	Yingst (1976)
	Not studied	Yes	Great Palm Island, Australia	Uthicke & Karez (1999)
<i>Stichopus chloronotus</i>	Not studied	Yes	Lizard Island, Australia	Uthicke (1999)
	Not studied	Yes	Great Barrier Reef, Australia	Moriarty (1982)
<i>Stichopus japonicus</i>	Not studied	Yes	Aquarium experiment, Japan	Michio <i>et al.</i> (2003)
<i>Stichopus tremulus</i>	Yes, coarse particles	Yes	Raunefjorden, Norway	Hauksson (1979)
<i>Stichopus variegatus</i>	Not studied	Yes	Great Palm Island, Australia	Uthicke & Karez (1999)
<i>Deep-sea species</i>				

> **Apodida**

<i>Chiridota</i> sp.	Not studied	No	Santa Catalina Basin and Hawaiian slope	Miller <i>et al.</i> (2000)
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> **Elasipodida**

<i>Amperima rosea</i>	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Wigham <i>et al.</i> (2003)
	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Ginger <i>et al.</i> (2001)
<i>Benthogone rosea</i>	Yes, 7-14 μ m	Yes	Golfe de Gascogne, France	Khripounoff & Sibuet (1980)
<i>Ellipinion molle</i>	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Ginger <i>et al.</i> (2001)
<i>Pannychia moseleyi</i>	Not studied	Yes	Santa Catalina Basin and Hawaiian slope	Miller <i>et al.</i> (2000)
<i>Peniagone vignoni</i>	Not studied	No	West Antarctic Peninsula	Wigham <i>et al.</i> (2008)
<i>Protelpidia murrayi</i>	Not studied	No	West Antarctic Peninsula	Wigham <i>et al.</i> (2008)

<i>Psychropotes longicauda</i>	Yes, 6.2-44 µm	Yes	Golfe de Gascogne, France	Khripounoff & Sibuet (1980)
	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Wigham <i>et al.</i> (2003)
<i>Scotoplanes globosa</i>	Not studied	Yes	Santa Catalina Basin and Hawaiian slope	Miller <i>et al.</i> (2000)
> Holothuriida				
<i>Mesothuria carnosa</i>	Not studied	Yes	Santa Catalina Basin and Hawaiian slope	Miller <i>et al.</i> (2000)
> Molpadida				
<i>Molpadia blakei</i>	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Wigham <i>et al.</i> (2003)
	No	Not studied	Golfe de Gascogne, France	Khripounoff & Sibuet (1980)
<i>Molpadia musculus</i>	Not studied	No	West Antarctic Peninsula	Wigham <i>et al.</i> (2008)
> Persiculida				
<i>Paroriza pallens</i>	Yes, 8-54 µm	Yes	Golfe de Gascogne, France	Khripounoff & Sibuet (1980)
<i>Pseudostichopus villosus</i>	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Wigham <i>et al.</i> (2003)
<i>Pseudostichopus sp.</i>	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Wigham <i>et al.</i> (2003)
	Not studied	No	West Antarctic Peninsula	Wigham <i>et al.</i> (2008)
> Synallactida				
<i>Paelopatides retifer</i>	Not studied	No	Santa Catalina Basin and Hawaiian slope	Miller <i>et al.</i> (2000)
<i>Oneirophanta mutabilis</i>	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Witbaard <i>et al.</i> (2001)
	Not studied	Yes	Porcupine Abyssal Plain, NE Atlantic	Wigham <i>et al.</i> (2003)

(a) Size selection of particles

Whether deposit-feeding holothurians exhibit a preference for substrates with a specific grain size is still debated (Mercier *et al.*, 2000), with conflicting results available (**Table 11**) even within a single species. For example, Mezali & Soualili (2013) stated that *H. tubulosa* and *Holothuria (Roweothuria) poli* Dell Chiaje select a grain size ranging between 200 and 600 μm , whereas Massin & Jangoux (1976) found no size preference for these same species. Some authors conclude that holothurians are unselective regarding the size of ingested particles because ingested sediment particles tend to be very similar in size to those of the bottom sediment (Yingst, 1976; Sloan & von Bodungen, 1980; Hammond, 1982). Trefz (1958) noted that diverse holothurian species forage and subsist on different substrates, suggesting that they may focus their feeding on specific substrates. Of the 29 shallow-water species evaluated to date, 20 show grain size selectivity (**Table 11**); among the four deep-sea species studied, only *Molpadia blakei* (Théel) is unselective (**Table 11**; Khripounoff & Sibuet, 1980).

Particle-size selection seems to be species dependent: preferred grain size is 200–600 μm for *H. tubulosa* (Mezali & Soualili, 2013), 125–250 μm for *H. scabra* (Baskar, 1994), and 2000–3500 μm for *H. nobilis* (Roberts & Bryce, 1982). Deep-sea species seem to prefer finer particles than shallow-water species, with a median of 7–14 μm for *Benthogone rosea* Koehler and 8–54 μm for *Paroriza pallens* (Koehler) (Khripounoff & Sibuet, 1980). Particle-size selection may change within a species depending on the season: *Holothuria (Stauropora) hawaiiensis* Fisher and *Bohadschia vitiensis* (Semper) prefer finer particles during the spawning period and gravel and coarse particles during the rest of the year (Dar & Ahmad, 2006).

More information is required to assess the benefits of feeding on small-grain sediments for holothurians and the reasons why only some species are selective. Although the mechanisms by which a preferred substrate is selected are not understood, Roberts (1979) proposed that this could be associated with the morphology of the oral tentacles.

(b) Selection of organically rich particles

A wide range of holothurian species from tropical, shallow-water regions (Moriarty, 1982; Hammond, 1983; Uthicke, 1999; Uthicke & Karez, 1999), temperate seas (Hauksson, 1979; Amon & Herndl, 1991), and deep seas (Miller *et al.*, 2000; Wigham *et al.*, 2003, 2008) are known to select organically rich particles from the sediment (**Table 11**). These species seem to be attracted by sediments with high organic content (Yingst, 1982) and can differentiate and capture the preferred particles (Massin & Jangoux, 1976; Moriarty, 1982). Out of the 37 species studied, 29 prefer particles enriched in organic matter (**Table 11**). Both shallow-water and deep-sea species seem to be highly selective, with 17 out of 20 (85%) and 12 out of 17 species (70%), respectively, showing selectivity for organic matter (**Table 11**).

Generally, the organic matter content of the ingested sediment is much higher than in the sediment surrounding the animal (Hauksson, 1979). Khripounoff & Sibuet (1980), studying the selective feeding of four abyssal species (*Psychropotes longicauda* Théel, *P. pallens*, *B. rosea* and *M. blakei*), found that with concentrations of organic carbon and nitrogen in the foregut were four and six times greater, respectively, than concentrations in the local sediment.

Moriarty (1982) suggested that particle size selection in holothurian deposit-feeders might be explained by a non-uniform distribution of organic matter in sediments. Uthicke (1999) supported this hypothesis, arguing that a higher organic content is usually associated with smaller sediment grains with low specific gravity, which a richer host microflora (bacteria, microalgae, etc.), due to their higher surface area to volume ratio (Johnstone, Koop & Larkum, 1990). All studies, with one exception [for *Holothuria (Holothuria) stellati* Delle Chiaje (Mezali & Soualili, 2013)], that showed a preference for a particular grain size also found a preference for organically rich particles where this was investigated (**Table 11**). A relationship between the organic load and particle size could therefore explain the confusion between these two selective strategies of deposit-feeders.

(c) Patch selectivity

Patch selectivity describes the preference of a mobile organism to feed on patches in a heterogeneous environment (Uthicke & Karez, 1999). A patchy distribution of shallow-water holothurians, thought to be related to their feeding habits (Uthicke & Karez, 1999), has been described in ecosystems including Milne Bay Province and Torres Strait islands (Skewes *et al.*, 2002, 2006), the Gulf of Mannar in India (Asha *et al.*, 2015), New Caledonia (Purcell *et al.*, 2009), and most parts of the western Indian Ocean (Conand, 2008). For holothurians, the energetic cost of processing poor sediment is higher than the cost of moving to a more suitable feeding substrate (Mercier *et al.*, 1999). Mercier *et al.* (1999) found that the locomotive speed of *H. scabra* juveniles was higher on substrates with low organic matter content (71–331 cm day⁻¹) than on substrates of better quality (150–215 cm day⁻¹), reflecting an active search for organically rich sediments. Similar results were reported for *H. atra* and *S. variegatus* (now *S. herrmanni*), which move 0–52 m per day and cover greater distances on poorer feeding areas (Yamanouchi, 1939). Other studies on *Cucumaria frondosa* (Gunnerus) (Hamel & Mercier, 1998), *P. californicus* (now *A. californicus*) (Cameron & Fankboner, 1984), *Parastichopus chitonoides* (Young & Chia, 1982), *Actinopyga echinites* (Jaeger) (Wiedemeyer, 1994), and *H. scabra* (James & James, 1994; Altamirano *et al.*, 2017) generally describe the quality of the substrate where holothurians were observed but do not detail optimal substrates in terms of nutritional or ecological features (e.g. optimal grain size, organic matter content, origin of sediments, presence of conspecifics or optimal light regime).

A patchy distribution also has been observed in deep-sea ecosystems for several species (Billett, Llewellyn & Watson, 1988; Ruhl & Smith, 2004) that feed preferentially on nutritionally rich food patches (Hauksson, 1979; Hudson *et al.*, 2005; Jamieson *et al.*, 2011). Particulate organic carbon deposited on the seabed is considered an important factor controlling the local abundance and composition of macrofauna and megafauna (Sibuet, 1985; De Leo *et al.*, 2010). Significant correlations have been found between food availability and megafaunal abundance, particularly for holothurians (Billett *et al.*, 2001; Ruhl & Smith, 2004). Preferences for selective feeding amongst holothurian species may therefore function in niche

partitioning (Roberts, 1979; Sloan & von Bodungen, 1980; Massin & Doumen, 1986), with reduced intra- and inter-specific competition as a result (Jamieson *et al.*, 2011).

IV. THE SEDIMENT INGESTED BY HOLOTHURIANS: A COMPLEX FOOD SOURCE

Despite much published work on holothurians, very few studies have focused on the quality of their food. **Table 12** provides a list of known food sources for holothurians, of both organic and inorganic origin. We divide these food sources into two categories: living and non-living fractions. Living fractions include organisms associated with sediments such as bacteria, photosynthetic organisms or meiofauna. Non-living fractions include organically derived detrital matter such as phytodetritus, dead and decaying animals, and faecal pellets and inorganic compounds such as coral scraps, shell remains, coralline algae, foraminiferal tests, and silicates. Sadly, holothurians have not escaped the increasing pollution of the ocean, with ingestion of plastic particles or microplastics recorded (Graham & Thompson, 2009; Renzi *et al.*, 2018).

Table 12: Food sources recorded as ingested by coastal and deep-sea holothurians.

Food sources ingested	Indicator studied	Authors	Order	Species studied	Habitat	Number of individuals studied
Living fractions						
Bacteria	Abundance	Deming & Colwell (1982)	Synallactida	<i>Deima sp.</i>	Deep sea	2
			Persiculida	<i>Pseudostichopus sp.</i>	Deep sea	3
		Roberts <i>et al.</i> (2001)	Persiculida	<i>Molpadiodemas villosus</i>	Deep sea	Up to 25
			Synallactida	<i>Oneirophanta mutabilis</i>	Deep sea	Up to 25
			Elasipodida	<i>Psychropotes longicauda</i>	Deep sea	Up to 25
		Taddéi (2006)	Holothuriida	<i>Holothuria atra</i>	Coastal	18
	<i>Holothuria leucospilota</i>			Coastal	7	
	Abundance and diversity	Amaro <i>et al.</i> (2012)	Molpadida	<i>Molpadia musculus</i>	Deep sea	20
		Plotieau <i>et al.</i> (2013)	Holothuriida	<i>Holothuria scabra</i>	Coastal	4 for abundance 30 for diversity
				<i>Holothuria atra</i>	Coastal	2
	Diversity	Amaro <i>et al.</i> (2009)	Molpadida	<i>Molpadia musculus</i>	Deep sea	15
		Gao <i>et al.</i> (2017)	Synallactida	<i>Apostichopus japonicus</i>	Coastal	240
		Sha <i>et al.</i> (2016)	Synallactida	<i>Apostichopus japonicus</i>	Coastal	30
		Zhang <i>et al.</i> (2012)	Holothuriida	<i>Holothuria leucospilota</i>	Coastal	2
Photosynthetic organisms	Abundance	Taddéi <i>et al.</i> (2006)	Holothuriida	<i>Holothuria atra</i>	Coastal	18
				<i>Holothuria leucospilota</i>	Coastal	6
	Abundance and diversity	Belbachir & Mezali (2018)	Holothuriida	<i>Holothuria forskali</i>	Coastal	10
				<i>Holothuria poli</i>	Coastal	10
				<i>Holothuria sanctori</i>	Coastal	10
<i>Holothuria tubulosa</i>	Coastal	10				

		Hamel & Mercier (1998)	Dendrochirotida	<i>Cucumaria frondosa</i>	Coastal	20
		Hamel <i>et al.</i> (1993)	Dendrochirotida	<i>Psolus fabricii</i>	Coastal	30
		Kang <i>et al.</i> (2008)	Holothuriida	<i>Holothuria atra</i>	Coastal	NA
			Elasipodida	<i>Benthogone rosea</i>	Deep sea	NA
		Khripounoff & Sibuet (1980)		<i>Psychropotes longicauda</i>	Deep sea	NA
			Molpadida	<i>Molpadia blakei</i>	Deep sea	NA
			Persiculida	<i>Paroriza pallens</i>	Deep sea	NA
		Sonnenholzner (2003)	Holothuriida	<i>Holothuria theeli</i>	Coastal	200
		Tyler <i>et al.</i> (1992)	Persiculida	<i>Paroriza pallens</i>	Deep sea	52
		Uthicke (1999)	Holothuriida	<i>Holothuria atra</i>	Coastal	6
			Synallactida	<i>Stichopus chloronotus</i>	Coastal	6
Meiofauna	Abundance and diversity			<i>Holothuria forskali</i>	Coastal	10
		Belbachir & Mezali (2018)	Holothuriida	<i>Holothuria poli</i>	Coastal	10
				<i>Holothuria sanctori</i>	Coastal	10
				<i>Holothuria tubulosa</i>	Coastal	10
		Hamel & Mercier (1998)	Dendrochirotida	<i>Cucumaria frondosa</i>	Coastal	20
		Kang <i>et al.</i> (2008)	Holothuriida	<i>Holothuria atra</i>	Coastal	NA
			Elasipodida	<i>Benthogone rosea</i>	Deep sea	NA
		Khripounoff & Sibuet (1980)		<i>Psychropotes longicauda</i>	Deep sea	NA
			Molpadida	<i>Molpadia blakei</i>	Deep sea	NA
			Persiculida	<i>Paroriza pallens</i>	Deep sea	NA
		Sonnenholzner (2003)	Holothuriida	<i>Holothuria theeli</i>	Coastal	200
		Tyler <i>et al.</i> (1992)	Persiculida	<i>Paroriza pallens</i>	Deep sea	52
		Uthicke (1999)	Holothuriida	<i>Holothuria atra</i>	Coastal	6
			Synallactida	<i>Stichopus chloronotus</i>	Coastal	6

Non-living fractions						
Detrital matter	Abundance	Suchaneck <i>et al.</i> (1984)	Holothuriida	<i>Mesothuria verrilli</i>	Deep sea	5
			Elasipodida	<i>Benthodytes lingua</i>	Deep sea	2
				<i>Psychropotes semperiana</i>	Deep sea	1
	Abundance and diversity	Belbachir & Mezali (2018)	Holothuriida	<i>Holothuria forskali</i>	Coastal	10
				<i>Holothuria poli</i>	Coastal	10
				<i>Holothuria sanctori</i>	Coastal	10
				<i>Holothuria tubulosa</i>	Coastal	10
		Costa <i>et al.</i> (2014)	Holothuriida	<i>Holothuria tubulosa</i>	Coastal	3
		Khripounoff & Sibuet (1980)	Elasipodida	<i>Benthogone rosea</i>	Deep sea	NA
	<i>Psychropotes longicauda</i>			Deep sea	NA	
Molpadida	<i>Molpadia blakei</i>			Deep sea	NA	
Persiculida	<i>Paroriza pallens</i>	Deep sea	NA			
Diversity	Hammond & Wilkinson (1985)	Apodida	<i>Synaptula lamperti</i>	Coastal	40	
Minerals	Abundance and diversity	Belbachir & Mezali (2018)	Holothuriida	<i>Holothuria forskali</i>	Coastal	10
				<i>Holothuria poli</i>	Coastal	10
				<i>Holothuria sanctori</i>	Coastal	10
				<i>Holothuria tubulosa</i>	Coastal	10
		Khripounoff & Sibuet (1980)	Elasipodida	<i>Benthogone rosea</i>	Deep sea	NA
				<i>Psychropotes longicauda</i>	Deep sea	NA
				Molpadida	<i>Molpadia blakei</i>	Deep sea
	Persiculida	<i>Paroriza pallens</i>	Deep sea	NA		
Tyler <i>et al.</i> (1992)	Persiculida	<i>Paroriza pallens</i>	Deep sea	52		
Diversity	Plotieau (2012)	Holothuriida	<i>Holothuria scabra</i>	Coastal	4	
Plastic particles	Graham & Thompson (2009)	Dendrochirotida	<i>Cucumaria frondosa</i>	Coastal	42	

Abundance and diversity			<i>Thyonella gemmata</i>	Coastal	30
		Holothuriida	<i>Holothuria grisea</i>	Coastal	46
			<i>Holothuria floridana</i>	Coastal	53
	Iwalaye <i>et al.</i> (2020)	Holothuriida	<i>Holothuria cinerascens</i>	Coastal	20
	Moshen <i>et al.</i> (2019)	Synallactida	<i>Apostichopus japonicus</i>	Coastal	65
	Renzi <i>et al.</i> (2018)	Holothuriida	<i>Holothuria tubulosa</i>	Coastal	30

(1) The living fractions

(a) Bacteria

The total biomass of bacteria is relatively high in shallow-water and deep-sea sediments (Zobell & Morita, 1959; Danovaro, Fabiano & Della Croce, 1993; Danovaro *et al.*, 1998; Rex *et al.*, 2006). Most benthic bacteria are not suspended in interstitial water but are attached to mineral or organic sediment particles (Dale, 1974) in aggregates and colonies (Taddéi, 2006). Thus, bacteria could represent a major food source for deposit-feeders (Sorokin, 1972; Massin, 1982) such as holothurians. We found only 10 articles that studied bacteria in the diet of deposit-feeding holothurians, of which four focused on the diversity of ingested bacteria, three on their abundance, and three on both. To our knowledge, the abundance and diversity of bacteria in the gut of suspension-feeding holothurians has not been assessed.

Most studies found a similar distribution pattern of bacteria in the digestive tract for coastal (Taddéi, 2006; Plotieau *et al.*, 2013) and deep-sea holothurian species (Deming & Colwell, 1982; Roberts *et al.*, 2001; Amaro *et al.*, 2012): an increase in bacterial abundance between the sediment and the foregut contents, then a decrease between the foregut and the hindgut. For example, Taddéi (2006), studying two coastal species, *H. atra* and *Holothuria (Mertensiothuria) leucospilota* (Brandt), found an abundance of 1.50×10^7 bacteria g^{-1} in coral reef shallow-water sediments of La Réunion. This increased to 3.66×10^7 bacteria g^{-1} in the foregut of *H. atra* and 4.46×10^7 bacteria g^{-1} in the foregut of *H. leucospilota*, which decreased significantly to 1.10×10^7 and 1.01×10^7 bacteria g^{-1} , respectively, in the hindgut. Bacterial abundance was similar in the faeces (1.91×10^7 and 0.87×10^7 bacteria g^{-1}). Taddéi (2006) reported that about 53% of bacteria are digested by *H. atra*. Plotieau *et al.* (2013) reported a higher abundance of 11×10^9 bacteria g^{-1} in the foregut of the coastal species *H. scabra*, which again decreased in the midgut (4×10^9 bacteria g^{-1}) and remained stable in the faeces. They estimated that *H. scabra* digested up to 59% of the ingested bacteria. Amaro *et al.*, 2012) reported a value of 80% for *Molpadia musculus* Risso.

Ward-Rainey, Rainey & Stackebrandt (1996) found the opposite pattern for the two specimens of *H. atra* that they studied. The abundance of bacteria decreased from 3×10^4 and 3×10^6 colony forming units (cfu) to 3.4×10^3 and 6.2×10^4 cfu, respectively, between the sediment and the foregut, and then increased to 3×10^4 and 1.8×10^6 cfu in the hindgut. However, the low number of replicates means that these results should be considered with caution.

All deposit-feeding holothurian species studied to date regarding the abundance of bacteria have peltate tentacles, except *M. musculus*, which has digitate tentacles (Amaro *et al.*, 2012). No quantitative difference in the ingestion of bacteria has been observed between species with these two tentacle types (**Figure 20**). No studies to date have focused on species in Apodida and Dendrochirotida (**Table 12**). Apodida with pinnate (highly branched) tentacles and suspension-feeding Dendrochirotida with dendritic (ultra-branched) tentacles are likely to show different ingestion rates of bacteria due to their higher tentacle surface area or different feeding behaviour.

Our understanding of the profiles of bacterial abundance in holothurian guts is based on only nine species, and 131 individuals, and additional data on the ingestion of bacteria by holothurians are needed.

Deming & Colwell (1982) suggested that holothurians can enhance the value of ingested bacteria by cultivating them in the foregut. Some bacteria may inhabit internal pouches or be attached to the gut epithelium (Harris, 1993). These enteric bacterial strains could help holothurians to digest large molecules through their secretion of hydrolytic ectoenzymes (Roberts *et al.*, 1991). Amaro *et al.* (2012) studied bacterial diversity through the digestive tract of *M. musculus*. They found that bacterial diversity showed the same pattern as bacterial biomass: there was a higher number of operational taxonomic units (OTUs) in the foregut (< 100 OTUs) than in sediment (28–71 OTUs), and bacterial diversity decreased towards the hindgut (< 70 OTUs). These observations may highlight the ability of holothurians to cultivate particular strains of bacteria in their foregut. As some endosymbiotic bacterial production could be involved, this requires further investigation.

Only a few studies have explored bacterial diversity in the holothurian gut. Five species have been investigated to date, including one deep-sea species. All these studies found Proteobacteria to be the most abundant taxon (**Figure 20**), representing 43% of bacteria in the diet of *H. leucospilota* (Zhang *et al.*, 2012), 86% in *H. scabra* (Plotieau *et al.*, 2013), and 55% (Sha *et al.*, 2016) and 89.6% (Gao *et al.*, 2017) in *A. japonicus*. The forms ingested most were γ -proteobacteria and α -proteobacteria for all holothurian species studied. β -proteobacteria, δ -proteobacteria and ϵ -proteobacteria were also present in some species.

In coastal species, 15 taxa of bacteria were identified, whereas only seven taxa were found in deep-sea species. Five of these taxa (γ -proteobacteria, α -proteobacteria, CFB lineage, Bacteroidetes and Spirochaetes) were shared between coastal and deep-sea species, perhaps because the diversity of deep-sea bacteria is lower than the diversity of coastal bacteria.

Knowledge on the abundance and diversity of bacteria is based on the study of less than 500 specimens from only 10 species: four from coastal waters and six from the deep sea belonging to Elaspodida, Persiculida, Holothuriida, Molpadida and Synallactida (**Table 12**). To the extent that these limited findings allow, it can be concluded that holothurians ingest mainly proteobacteria and show a similar distribution of bacteria through the gut across holothurian species, habitat and tentacle type (**Figure 20**).

(b) Photosynthetic organisms

Holothurians also consume photosynthetic organisms from both the microphytobenthos (including benthic diatoms, cyanophytes, dinoflagellates, etc.) and macrophytes (macroalgae and phanerogams) for deposit-feeding holothurians, and phytoplankton (including pelagic diatoms, cyanobacteria, dinoflagellates, etc.) for suspension-feeding holothurians.

The microphytobenthos in shallow-water sediments is mainly dominated by diatoms (Uthicke & Klumpp, 1998; Suzumura *et al.*, 2002; Heil *et al.*, 2004). Taddéi (2006) recorded a mean abundance of 933 diatom cells g^{-1} in the sediment of Reunion Island. In deep-sea sediments, diatoms are also at surprisingly high concentrations (Wood, 1956; Van Iperen *et*

al., 1987; Thiel *et al.*, 1989), with high abundances of cyanophytes and dinoflagellates associated with deposited phytodetritus (Lochte & Turley, 1988; Thiel *et al.*, 1989).

Nine studies have analysed the importance of photosynthetic organisms in the diet of holothurians (**Table 12**): of which seven focused on 10 coastal species (Hamel, Himmelman & Dufresne, 1993; Hamel & Mercier, 1998; Uthicke, 1999; Sonnenholzner, 2003; Taddéi, 2006; Kang *et al.*, 2008; Belbachir & Mezali, 2018) and two studies on four deep-sea species (Khripounoff & Sibuet, 1980; Tyler *et al.*, 1992).

The most abundant photosynthetic taxa in the diet of holothurians seems to be diatoms (**Figure 20**). Hamel *et al.* (1993) analysed the gut contents of *Psolus fabricii* (Düben & Koren), a suspension-feeding species, and found less than 6000 pelagic diatom cells in the first centimeter of the foregut. Diatoms were recorded at much higher concentrations in the gut of *Holothuria (Selenkothuria) theeli* Deichmann (up to 16,500 organisms ml⁻¹; Sonnenholzner, 2003). For the deep-sea species investigated, the sighting frequency of diatoms in *P. longicauda*, *P. pallens*, *B. rosea*, and *M. blakei* gut ranges between 51 and 84% (Khripounoff & Sibuet, 1980). Tyler *et al.* (1992) also found a high abundance of diatoms in the gut of *P. pallens*. However, these studies provide no information on whether the diatoms were alive or whether only the siliceous outer skeleton was present.

Photosynthetic organisms may be significant in the diet of holothurians. Fresh organic matter provided by cyanophytes, diatoms, macroalgae and live seagrass leaves represents more than 50% of the diet for *H. poli*, *H. tubulosa*, and *H. forskali* in *Posidonia oceanica* meadows in Algeria (Belbachir & Mezali, 2018). In shallow waters off Ecuador, 35% of the gut content of *H. theeli* consisted of microphytobenthos, with the most dominant taxa being diatoms (91%), followed by cyanophytes and dinoflagellates (Sonnenholzner, 2003). However, these studies did not assess all the possible dietary fractions for holothurians, such as bacteria or minerals, therefore these high proportions may be significant overestimates.

Hamel *et al.* (1993) estimated that phytoplanktonic cells represent between 20 and 50% of the gut content of *P. fabricii* in autumn and winter, increasing during spring to reach 100% in summer in the St. Lawrence Estuary (Canada). They conclude that the ingestion of

phytoplanktonic cells, especially pelagic diatoms ingested by this dendrochirotidan holothurian (suspension feeder), depends on seasonal blooms. Similar results were observed for *C. frondosa*, with the proportion of phytoplanktonic cells ingested reaching a maximum in summer (Hamel & Mercier, 1998). Sonnenholzner (2003) investigated the ingestion of photosynthetic organisms in a coastal deposit-feeding species from Holothuriida, finding that the relative abundance of microphytobenthic species in the *H. theeli* gut was significantly higher during the dry season (45.5%) than in the rainy season (20.2%) in the Gulf of Guayaquil. It would be interesting to extend these findings to all fractions present in the holothurian diet to understand which components replace photosynthetic organisms during the cold seasons.

Taddéi (2006) used the concentration of chlorophyll a as proxy for photosynthetic organisms in the gut for two coastal deposit-feeders, *H. atra* and *H. leucospilota*. The profile observed was the same as for bacterial abundance: chlorophyll a concentration increased from the local sediment ($2.87 \pm 1.17 \mu\text{g g}^{-1}$) to the foregut ($18.74 \pm 7.65 \mu\text{g g}^{-1}$), decreased in the hindgut ($11.28 \pm 4.61 \mu\text{g g}^{-1}$), and remained at this level in the faeces ($11.34 \pm 4.63 \mu\text{g g}^{-1}$). These observations may indicate that photosynthetic organisms are actively selected for ingestion by these deposit-feeding holothurians.

These studies combined used 378 individuals from six orders of holothurians: Dendrochirotida, Holothuriida, Synallactida, Molpadida, Elasipodida and Persiculida (**Table 12**). Benthic and pelagic diatoms are a significant dietary component for deposit-feeding and suspension-feeding holothurians respectively, independent of habitat, taxon and tentacle type (**Figure 20**). However, the importance of photosynthetic organisms remains unclear without further studies because their biomass is subject to seasonal variations.

(c) Meiofauna

Meiofauna densities in shallow-water sediments range from 3.2 to 1,020.6 individuals 10 cm^{-2} (Guzman, Obando & Cortés, 1987; Armenteros, Creagh & González-Sansón, 2009). In deep-sea sediments, meiofauna densities are lower than in shallow waters, ranging from 15 to 315 individuals 10 cm^{-2} (Coull *et al.*, 1977; Pfannkuche, 1985). In both ecosystems,

nematodes and foraminifera are the dominant taxa (Coull *et al.*, 1977; Pfannkuche, 1985; Guzman *et al.*, 1987; Armenteros *et al.*, 2009).

Only seven studies have considered the ingestion of meiofauna by holothurians (**Table 12**). Nematoda, Copepoda and Foraminifera seem to be the taxa ingested most frequently by holothurians although there are interspecific differences. *Holothuria atra* ingests up to 79% of the meiofauna present in the sediment, with polychaetes the dominant group (Kang *et al.*, 2008). Dissection of *H. theeli* individuals showed that 65% of the gut contents consisted of meiofauna from eight taxa, with a dominance of crustaceans (46%) and foraminifera (35.5%) (Sonnenholzner, 2003). Belbachir & Mezali (2018) demonstrated that the percentage contribution of meiofauna in the holothurian diet can differ among locations and species. They studied the diet of four species *H. poli*, *H. tubulosa*, *H. forskali*, and *Holothuria (Platyperona) sanctori* Dell Chiaje, at two sites (Stidia and Salamandre in Algeria; separated by less than 20 km). At Salamandre, 20% of the diet of *H. sanctori* consisted of crustaceans, whereas in Stidia crustaceans comprised less than 3%. For the three other species, crustaceans represented only 6% of the diet at Salamandre. The contribution of foraminifera ranged between 3.33% for *H. sanctori* and 15% for *H. forskali* at the same site.

For the deep-sea species *P. longicauda*, *P. pallens*, and *B. rosea*, the sighting frequency of benthic foraminifera reached 100% of the gut content and was 89% for *M. blakei* (Khripounoff & Sibuet, 1980). The sighting frequency of Nematoda and Copepoda was also high for these four species (96–100% and 0–87%, respectively). Tyler *et al.* (1992) recorded abundant foraminifera in the gut of *P. pallens* but no Nematoda nor Copepoda. Note that the sighting frequency is a poor indicator of the actual contribution of meiofauna to the diet.

Uthicke (1999) compared the abundances of Nematoda, Polychaeta and Harpacticoida between the sediment and the midgut content of *S. chloronotus* and *H. atra*. Nematoda were the dominant taxon in the sediments (31.33 ± 10.90 individuals 2 ml^{-1}), but they were sparsely represented in the midgut of these two species (between 0.50 ± 0.84 and 0.66 ± 1.03 individuals 2 ml^{-1}). Polychaeta were more uncommon in the sediment (9.66 ± 5.31 individuals 2 ml^{-1}) and in the midgut of *S. chloronotus* and *H. atra* (0.17 ± 0.41 and

0.01 ± 0.00 individuals 2 ml⁻¹ respectively). Uthicke (1999) concluded that meiofauna play a negligible role in the nutrition of these two species due to the extremely low abundance recorded in the midgut. The ingestion of these two meiofaunal taxa appears to be in proportion to their presence in the sediment, suggesting that meiofauna are probably ingested accidentally with detrital matter (Khripounoff & Sibuet, 1980; Billett *et al.*, 1988).

One study focused on the ingestion of larval planktonic stages of meiofauna by a suspension-feeding holothurian. Meiofaunal eggs and embryos accounted for less than 15% of the intestinal content of *C. frondosa* in autumn and winter (Hamel & Mercier, 1998). Maximum zooplankton presence peaked just after breeding of broadcast-spawning species. The actual contribution of meiofauna to the diet of suspension-feeding holothurian thus remains largely unknown. As for photosynthetic organisms, the availability of larval planktonic stages of meiofauna varies seasonally and suspension-feeding holothurians that ingest larval planktonic stages of meiofauna in proportion to their presence in the water column must utilise other resources in the autumn and winter in cold or temperate waters. This implies that meiofauna are not an essential dietary component for holothurians.

The ingestion of meiofauna has been investigated in all holothurian orders except Apodida (**Table 12**). The meiofauna ingested differs between deposit-feeding and suspension-feeding holothurians (Dendrochirotida), because the latter ingest larval planktonic stages of meiofauna. However, for the other orders Foraminifera, Nematoda, Polychaeta and Crustacea are all ingested at a similarly low rate, independent of tentacle or habitat type (**Figure 20**).

(2) Non-living fractions

(a) Detrital matter

Very few studies are known to feed on detrital matter (**Table 12**) ingested by coastal and deep-sea holothurians. Shallow-water species can feed on detrital matter from seagrasses or algae. Massin & Jangoux (1976) recorded *H. tubulosa* feeding on detrital seagrass leaves and Costa, Mazzola & Vizzini (2014) reported a population of *H. tubulosa* to ingest 30–100% of the detritus of *P. oceanica* meadows, depending on holothurian density. Dead *Posidonia*

leaves comprise 0.66–14% of the gut content of *H. tubulosa* and *H. forskali*, respectively (Belbachir & Mezali, 2018). Deep-sea ecosystems also receive phytodetritus, such as dead leaves of seagrasses or algae (Inman & Frautschy, 1965). Using stable isotope analyses, Suchanek *et al.* (1985) reported that at least two deep-sea species, *Mesothuria verrilli* (Thée) and *Benthodytes lingua* Perrier R., feed on sediments enriched by decaying seagrasses and consume and metabolise seagrass detritus. Khripounoff & Sibuet (1980) estimated that *P. pallens*, *M. blackei*, *B. rosea* and *P. longicauda* feed on macrophytic detritus, although with a low sighting frequency of 13%. Together these observations indicate that detrital matter from macrophytes, in both coastal and deep-sea species is ingested relatively rarely (**Figure 20**).

In the deep sea, only a small fraction of macroaggregates originating from the euphotic zone reaches the seabed to form detrital matter, with a low increment of 100–150 m day⁻¹ (Gooday & Turley, 1990), limiting their accessibility for holothurians (Thurston *et al.*, 1994; Thurston, Rice & Bett, 1998). Most of this fraction is faecal matter, which represents 95% of vertical particle flow (Wiebe, Boyd & Winget, 1976; Honjo, 1978) and constitutes a key component of the abyssal food web (Frankenberg & Smith, 1967). Holothurians are known to be coprophagous (Bakus, 1973; Hauksson, 1979). However, it remains unclear whether coprophagous holothurians feed on faecal pellets intentionally or simply take advantage of a proximate source of concentrated nutrients. Faecal pellets were found in the gut of all specimens of *P. longicauda*, *P. pallens*, *B. rosea*, and *M. blackei* examined (Khripounoff & Sibuet, 1980). These faecal pellets were from bivalves, pelagic crustaceans, and unidentified sources. Faecal pellets have not been recorded in the diet of coastal species.

Several studies have suggested that detrital matter is a major dietary component for both shallow-water and deep-sea holothurian species (Bordovskiy *et al.*, 1974; Yingst, 1976; Massin, 1982; Moriarty, 1982; Jeffrey *et al.*, 2011). Plotieau (2012) focused on the assimilation of organic compounds from seagrass phytodetritus by *H. scabra*. Experiments showed that *H. scabra* assimilated organic compounds from seagrass leaves, however, this assimilation appeared insufficient to support juvenile growth. Plotieau (2012) therefore suggested that the ingestion of seagrass detritus could be related to the heterotrophic

bacteria and microautotrophs attached to them. Indeed, detrital matter, such as phytodetritus, is mainly degraded by bacteria, which colonise it rapidly in both coastal and deep-sea ecosystems (Fenchel & Jorgensen, 1977; Lochte & Turley, 1988; Thiel *et al.*, 1989; Kaiser & Benner, 2008). If correct, this suggests that ingestion of detrital material by holothurians may represent an opportunistic behaviour, to access the bacteria attached to it, rather than detritivorous feeding *per se*.

The ingestion of detrital matter has been relatively poorly studied, with fewer than 100 individuals analysed (**Table 12**). No studies have focused on Dendrochirotida (suspension feeders) nor Synallactida. Deep-sea species ingest faecal pellets while coastal species do not. Overall, detrital matter may not be a significant fraction in the diet of holothurians, with the small quantities ingested probably more linked to the presence of bacteria.

(b) Minerals

The non-organic fractions ingested by holothurians are receiving increasing attention, with innovative work suggesting an important role in feeding efficiency and nutritional benefits (Plotieau, 2012).

The sediments ingested by deposit-feeding holothurians contain insoluble clastic products originating from physical and biological breakdown. An analysis of minerals in the sediment ingested by tropical shallow-water holothurians identified both primary and secondary minerals (Plotieau, 2012). The primary minerals were (1) quartz made up of a continuous framework of SiO_4 (identified as the main mineral holothurian gut component in most studies), (2) calcite, the most stable polymorph of calcium carbonate (CaCO_3), (3) aragonite, a thermodynamically unstable form of CaCO_3 at standard temperature and pressure, and (4) magnesian calcite (Ca, MgCO_3), a variety of calcite containing randomly substituted magnesium carbonate in a disordered calcite lattice that is present in echinoderm skeletons. The secondary minerals were (1) bioclasts, skeletal fragments of marine or land organisms found in sedimentary rocks (mainly composed of aragonite but also magnesian calcite and calcite), (2) feldspars (KAlSi_3O_8 , $\text{NaAlSi}_3\text{O}_8$, $\text{CaAl}_2\text{Si}_2\text{O}_8$), a group of rock-forming tectosilicate minerals, (3) hornblende or dark amphibole, an isomorphous mixture of calcium–

iron–magnesium silicate, aluminium–iron–magnesium silicate, and iron–magnesium silicate), and (4) other, trace minerals, mainly zircon.

Three studies analysed the gut contents of shallow-water or deep-sea species with respect to minerals (**Table 12**; Khripounoff & Sibuet, 1980; Tyler *et al.*, 1992; Belbachir & Mezali, 2018). They identified the presence of mollusc, echinoderm, and sponge ossicles along with various pelagic and benthic foraminifera tests. The mineral fraction in the diets of four shallow-water species can exceed 30%, with less than 12% bivalve shells and 12–24% sponge ossicles (Belbachir & Mezali, 2018). A high sighting frequency of 59–79% was reported for spicules and 100% for coccoliths, in the diets of four deep-sea species (*P. longicauda*, *P. pallens*, *B. rosea*, and *M. blakei*; Khripounoff & Sibuet, 1980). Tyler *et al.* (1992) only rarely recorded spicules or ossicles in the gut of *P. pallens* although coccoliths were abundant.

(c) Plastic particles

Recent decades have revealed the impact of increasing plastic input into the ocean on marine taxa, such as fishes, seabirds, turtles, and cetaceans (Cole *et al.*, 2011), and how plastic particles penetrate the marine trophic web (Ivar do Sul & Costa, 2014). Species belonging to lower trophic levels can ingest high levels of plastic particles because they do not differentiate between these and their preferred food (Renzi *et al.*, 2018).

Several studies have recorded the ingestion of plastic particles (**Table 12**) in shallow-water deposit-feeding species (Graham & Thompson, 2009; Renzi *et al.*, 2018; Mohsen *et al.*, 2019) and suspension-feeding species (Graham & Thompson, 2009; Iwalaye, Moodley & Robertson-Andersson, 2020). *Cucumaria frondosa*, *Holothuria* (*Semperothuria*) *cinerascens* (Brandt), *Holothuria grisea* Selenka, *Holothuria floridana* (Pourtalès) and *H. tubulosa* were all shown to ingest plastic particles. Mohsen *et al.* (2019) found a lower abundance of plastic in the gut of farmed *A. japonicus* (0–30 particles per individual) than in the local sediments (20–1040 particles kg⁻¹). It is likely that most shallow-water species will ingest plastics if they are present in the sediment (Graham & Thompson, 2009). Recent studies show that, due to the vertical transport of particles from the sea surface to the sea floor, plastic particles are sequestered in deep-sea sediments from the Atlantic, Pacific, and Indian Oceans and the

Mediterranean Sea (Van Cauwenberghe *et al.*, 2013; Woodall *et al.*, 2014; Fischer *et al.*, 2015). Plastic particles have been found in the gut of other deep-sea benthic invertebrates including Cnidaria, Echinodermata, Arthropoda (Taylor *et al.*, 2016), and Mollusca (Courtene-Jones *et al.*, 2017). Thus, deep-sea holothurian species may also be exposed to and ingest plastics.

Mohsen *et al.* (2019) also found plastic particles in the coelomic fluid, ranging from 0 to 19 particles per individual. Iwalaye *et al.* (2020) found a similar result in tank experiments with *H. cinerascens* (32–227 microfibrils per individual). In more than half of the individuals studied (57.8%), plastic particles were present in the respiratory tree (0–12 microfibrils per individual). The biological impacts of the transfer of microplastic particles from the holothurian gut to the rest of the body were not analysed further.

Grossmann (2014) and Assidqi (2015) assessed the impact of the ingestion of plastic particles on *H. sanctori* and *H. leucospilota*, respectively. They exposed these holothurians to plastic particles followed by hypoxic conditions to investigate the effects of plastic particle ingestion on resistance to environmental stress. Ingestion of plastic particles did not affect faeces production, evisceration, respiration rate, survival, or behavioural responses in these two species. However, Assidqi (2015) reported a higher susceptibility of *H. leucospilota* to oxygen depletion 60 days after plastic particle ingestion.

Experiments in mesocosms revealed that holothurians appear preferentially to select plastic particles from the sediment (Graham & Thompson, 2009). The authors hypothesised that the larger surface area of the plastic particles reduces the need to shovel or rake for other particles leading to their selection in preference to sand grains. Similar results were found in a natural environment: analyses of sediment sieved through 63–4,000 μm sieves revealed that *H. tubulosa* selects 100–2,000 μm plastic fragments for ingestion (Renzi *et al.*, 2018).

When they reach shallow-water sediments, plastic particles can rapidly be colonised by bacteria (Harrison *et al.*, 2014). Dussud *et al.* (2018) demonstrated that bacterial abundance and diversity is higher on plastic particles than on organic particles. Bacterial communities found on the surface of plastic particles include Proteobacteria, Bacteroidetes (Oberbeckmann *et al.*, 2014; Curren & Leong, 2019), and Cyanobacteria (Oberbeckmann *et*

al., 2014; Dussud *et al.*, 2018). Other organisms, such as diatoms (Carson *et al.*, 2013; Eich *et al.*, 2015) and dinoflagellates (Masó *et al.*, 2003) can also be attached to plastic particles. All of these organisms are found in the holothurian diet. Finally, because holothurians select more bacteria-rich sediments, they could easily preferentially select plastic particles with bacteria attached, although this requires further investigation.

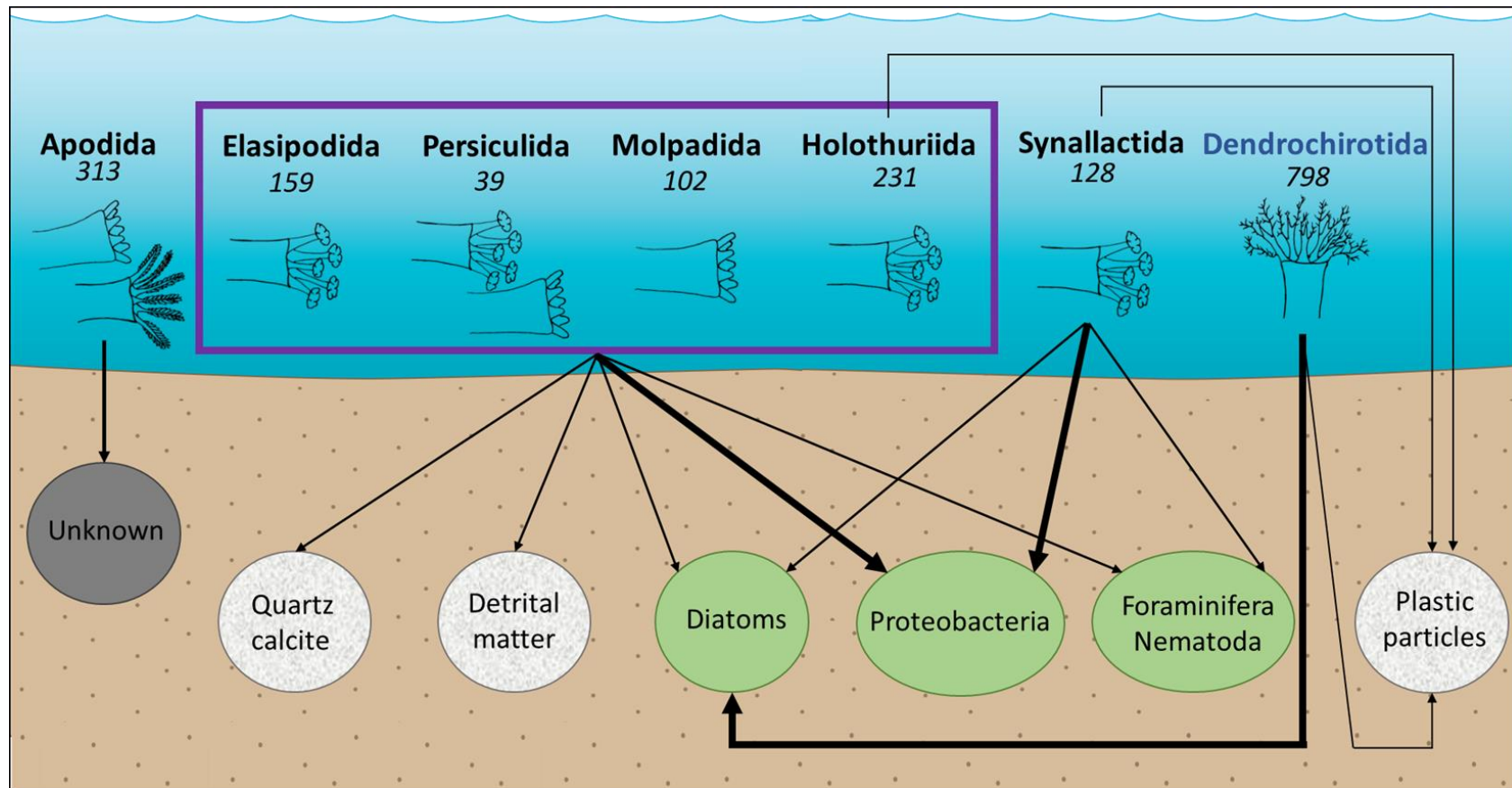


Figure 20: Synthesis of food sources for the seven orders of holothurians. Order colours represent different trophic modes: blue, suspension-feeders (*Dendrochirotida*); black, deposit-feeders. The number of species per taxon is shown below the order name. The width of arrows corresponds to the proportion of food ingested per source. Colours of food sources vary with food type: green, living fraction; grey, non-living fraction. Tentacle type for each taxon is represented by the drawings (see Fig. 2): digitate (unbranched); peltate (slightly branched); pinnate (highly branched); dendritic (ultra-branched).

V. CONCLUSIONS

(1) Cells on the holothurian tentacle bud epidermis are important for food capture. Ciliated cells are likely to be sensory cells that recognise food particles. Mucous cells may be responsible for the production of a physical barrier on the epidermis. The adhesion of food particles is suggested to be performed by the secretion of type-1 secretory cells; when the tentacles are placed in the oral cavity, adhesion may be removed by the secretion of type-2 secretory cells. Further studies are needed to confirm these hypothesised roles in holothurian taxa.

(2) Holothurian nutrition appears to involve two main feeding modes: some species are suspension-feeders whereas others are deposit-feeders, with a few species able to shift between suspension- and deposit-feeding. A third feeding pathway may exist, which relies on nutrient transfer from cloacal water retained by the animal to the haemal system.

(3) Synallactida, Molpadida, Persiculida, Holothuriida and Elasipodida, despite their different tentacle types and the different habitats they colonise, ingest similar proportions of different food types. Their diet seems to be composed mainly of proteobacteria. Diatom ingestion may be high, but its overall contribution remains uncertain as its availability varies seasonally. Other smaller fractions, such as detrital matter, minerals and plastic particles, may be ingested only because they support a high bacterial load.

(4) Dendrochirotida is the most recent order of holothurians. The species belonging to this order have dendritic tentacles that allow them to act as suspension-feeders. Most species in Dendrochirotida are coastal (more than 98%). Their trophic specialisation presumably reduces interspecific competition for food, and they ingest large quantities of pelagic diatoms. The ingestion of other food fractions has not yet been studied for Dendrochirotida.

(5) There is very little information on the ingestion of food by Apodida. This is a considerable knowledge gap because Apodida represent 17% of holothurian species. More than half of Apodida species have pinnate tentacles that are not present in other orders.

VI. ACKNOWLEDGMENTS

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The natural diet of tropical sea cucumbers: variability of bacteria communities in sediments and digestive tract

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Keywords

Sea cucumbers • Diet • Benthic bacteria • Nutritional process • Ingestion • Assimilation • Metabarcoding

Introduction

Sea cucumbers are marine invertebrates thriving in various ecosystems, such as coral reefs (Eriksson *et al.*, 2010) or abyssal trenches (Kuhnz *et al.*, 2014). Currently more than 1,700 species are described (WoRMS, 2023), but only two species have entered a complete process for large-scale aquaculture (Yang, Hamel & Mercier, 2015b; Hamel *et al.*, 2022). Feeding habits of sea cucumbers are well-described. They are either suspension-feeders or deposit-feeders. According to Roberts *et al.*, (2000), the deposit-feeding mode can be separated in three groups related to the foraging depth: epibenthic deposit-feeding at the interface between water column and substrate, subsurface deposit-feeding below the sediment surface and funnel deposit-feeding in funnel-depressions. Some sea cucumber species feed continuously, as *Holothuria edulis* or *Holothuria atra* (Yamanouchi, 1956; Trefz, 1958) whereas others exhibit a daily cycle for foraging, as *Holothuria scabra* and *Stichopus chloronotus* (Yamanouchi, 1956;

Klinger & Johnson, 1998). The ingestion rate and the time for sediment passing through gut have been estimated for several species, from 0.158 for *Leptosynapta inhaerens* (Manship, 1995 cited in Roberts *et al.*, 2000) to 200 g of sediment per day for *H. atra* (Trefz, 1958). The habitat preference related to food availability was highlighted for some species: for instance, *H. atra* prefers seagrass habitat with 2–3.5% of organic content (Dissanayake & Stefansson, 2012). The morphology of sea cucumbers has been well described. Different shapes of tentacles have been reported, from digitate (unbranched), peltate (slightly branched), pinnate (highly branched) to dendritic (ultra-branched) forms (see Pierrat *et al.*, 2022 for a review). Tentacles are the first body part in contact with the substrate, where particles of food are trapped. Then, the gut is divided in three parts (from mouth to anus): the foregut, the midgut and the hindgut, where absorption and assimilation occur (Massin, 1978). Bacteria are part of the diet and are studied (Deming & Colwell, 1982; Harris, 1993).

Paradoxically, despite the description of all these feeding characteristics, a knowledge gap still remains concerning the diet of sea cucumbers. According to review by Pierrat *et al.*, (2022), diet of sea cucumbers can be divided in two, with on one hand the living fraction, mainly constituted by bacteria, photosynthetic organisms and meiofauna, and on the other hand, the non-living fraction, including detrital matter and inorganic compounds. Even water column can be a source of food, containing nutrients and microorganisms (Pierrat *et al.* 22). Nowadays, no study has focused on all the fractions. The contribution of each fraction and selective processes remain poorly understood and no global snapshot of the diet have been realised. Regarding the living fraction, the bacteria component has received more attention, mostly for aquacultured species, *Apostichopus japonicus* (Gao *et al.*, 2014, 2017; Sha *et al.*, 2016; Bogatyrenko & Buzoleva, 2016; Wang *et al.*, 2018) and *Holothuria scabra* (Plotieau *et al.*, 2013) or some deep-sea species (Deming & Colwell, 1982; Amaro *et al.*, 2009, 2012). Ward-Rainey, Rainey & Stackebrandt, (1996) have examined the benthic bacteria communities of *H. atra*, but in two individuals only. Consequently, a gap of knowledge of benthic bacteria involved in the diet of wild coastal deposit-feeding sea cucumber populations exists.

Despite new sequencing technics offer a new insight on the benthic bacteria involved in the diet of sea cucumbers, some approximations subsist. In fact, most of the studies deal

with high taxonomic levels, as the Phylum, which is poorly informative regarding the high diversity of the benthic bacteria and provides little understanding about the diet of sea cucumbers. Additionally, they do not consider all of the compartments involved in the diet of sea cucumbers, namely the sediment beneath tentacles, the foregut, the hindgut and the faeces. Consequently, no global snapshot of the benthic bacteria can be obtained and no comprehension of the nutritional processes, as ingestion or assimilation, can be achieved. Finally, possible spatio-temporal variabilities of the benthic bacteria involved in the diet of sea cucumber are poorly explored.

About 40 species of sea cucumbers have been lately recorded in the coral reefs of Reunion Island (Conand *et al.*, 2018). Among these 40 species, three are patchily distributed and reach very high densities ($> 1 \text{ ind.m}^2$), *Holothuria atra*, *Holothuria leucospilota* and *Stichopus chloronotus*. Factors driving these aggregations have been recently investigated. The main mode of reproduction of *S. chloronotus* was the asexual reproduction by transversal fission (Conand *et al.*, 1998; Pirog *et al.*, 2017; Pierrat *et al.*, 2023). From a unique individual, this mode of reproduction conducted to two genetically identical individuals at the adult stage, who will remain close to each other. Conversely, no genetic evidence of asexual reproduction has been found for *H. atra* and *H. leucospilota*, meaning that the main mode of reproduction for these two species was sexual reproduction through gamete spawning, inside and outside of high-density patches (Pierrat *et al.*, 2023). This mode of reproduction leads to the release of gametes, which will migrate with the current. In consequence, larval stages can be found inside or outside the reef. The juvenile recruitment process is poorly known for sea cucumbers at Reunion Island, but some studies stated that juveniles do not use the same habitat and resources as adults (Mercier, Battaglione & Hamel, 2000a; Mercier *et al.*, 2000b). Consequently, asexual reproduction can be considered as a factor participating to the high-density aggregations observed, notably for *S. chloronotus* in Reunion Island. Anthropogenic factors of environmental stresses, such as pesticide pollution, seem to have no influence on the distribution of sea cucumbers in Reunion Island. Finally, the variability of sedimentary factors, such as substrate cover, total organic matter load or chlorophyll a concentration in relation with sea cucumber densities has been studied. However, the sedimentary factors monitored did not fully explain the patchy distribution observed (Chapter 3).

One of the main factors that can drive distribution of species is the food availability and quality, as already demonstrated for instance for right whale (Sorochan *et al.*, 2021), sea turtles (McCarthy *et al.*, 2010) or sea urchins (Lauzon-Guay & Scheibling, 2007). Some studies have also showed the relation between food availability and sea cucumber distribution (Bulteel *et al.*, 1992; Uthicke & Karez, 1999; Dissanayake & Stefansson, 2012). The relation between food and sea cucumber distribution in Reunion Island has been sparsely examined using CHN analysis. Mangion *et al.*, (2004) revealed that organic carbon and nitrogen concentrations increased between sediment and foregut of *H. leucospilota* or *H. atra*. The authors hypothesized that this rise was due to the presence of bacteria in the foregut.

The aim of this study is therefore to investigate the role of food in the patchy distribution observed of sea cucumbers in Reunion Island. More precisely, this study focuses on the diversity and spatio-temporal distribution of benthic bacteria included in the diet and its implication in the ingestion and assimilation nutritional processes in three species distributed along Reunion Island reefs (*Holothuria atra*, *Holothuria leucospilota* and *Stichopus chloronotus*). Finally, this study attempts to identify the major taxa of bacteria of the diet these sea cucumbers.

Materials & Methods

Study area

This study was conducted at Reunion Island (21°06'S, 55°31'E), located in the Western Indian Ocean and part of the hotspot of the Mascareignes, with Madagascar, Mauritius and Rodrigues. Reunion Island is a young volcanic island (Duncan *et al.*, 1989), under an austral tropical climate with two seasons, a warm and humid season from November to April, and a cold and dry season from May to October (Conand *et al.*, 2007). Four fringing reefs are found along the West and South coasts of the island: Hermitage/La Saline, Saint-Leu, Étang-Salé and Saint-Pierre, for a total area of 12 km². The first three reefs have been managed by the Réserve Nationale Marine de La Réunion, a marine protected area, since 2007. As sea cucumbers of Reunion Island have a patchy distribution (Pierrat *et al.*, submitted soon), seven sites were chosen across the reefs of Hermitage/La Saline and Étang-Salé: five with high-density patches

of sea cucumbers $> 1 \text{ ind.m}^{-2}$) and two low density sites ($< 0.01 \text{ ind.m}^{-2}$) distant from any high-density patch (**Figure 21**). From North to South in the reef of Hermitage/La Saline these sites are: “MNS” (Maître-Nageurs-Sauveteurs) with high density of *H. leucospilota*, “CAP” (Cap Méchant) with low density of *H. leucospilota*, “PAS” (Passe de l’Hermitage) with high density of *S. chloronotus*, “PLA” with high density of *H. leucospilota* and *H. atra*, “PTE” with low density of *H. leucospilota* and “TE” with high density of *H. leucospilota* and *S. chloronotus*. “ES” site is located in the reef of Étang-Salé, with high density of *S. chloronotus* only. Four of these sites (PAS, PLA, TE and ES) have been characterized as eutrophic, receiving nutrient through enriched freshwaters for decades (Cuet et al., 1988; Cuvillier, 2016, P. Cuet personal communication).

Sampling design

Sampling was carried out over three consecutive seasons, the austral winter 2019 (September), the austral summer 2020 (February) and the austral winter 2020 (September). For each season, 24 individuals per species were harvested at each high-density site and 12 at low-density site (DEAL permit N°2020-37; $N_{\text{Holothuria leucospilota}} = 288$; $N_{\text{Holothuria atra}} = 72$ and $N_{\text{Stichopus chloronotus}} = 216$) (**Figure 21**). Individuals were stored individually in sterile plastic zipper bags. Additionally, sediment samples were collected by coring beneath the tentacles of each collected individual with a 2 mL sterile Eppendorf. Finally, fresh cohesive faeces of each individual were collected and placed in individual sterile 40 mL plastic vials. All samples (individuals, sediments and faeces) were immediately placed, directly in the field, in dry ice (-80°C). In the lab, samples were kept in freezers, at -80°C before analyses.

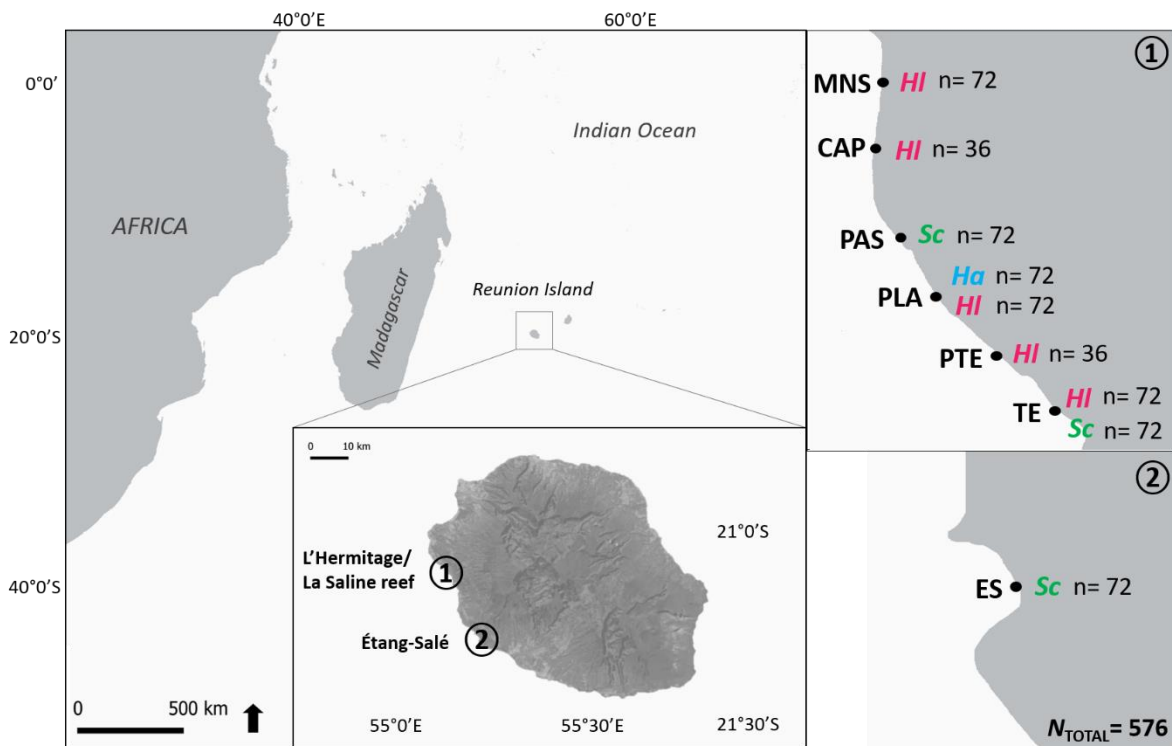


Figure 21: Location of Reunion Island in the Western Indian Ocean. 1) L'Hermitage/La Saline reef and 2) Étang-Salé reef, in the West coast of Reunion Island.

Name of sites are indicated in bold: MNS, CAP, PAS, PLA, PTE, TE and ES.

HI : *Holothuria leucospilota* in pink; *Ha* : *Holothuria atra* in blue; *Sc* : *Stichopus chloronotus* in green.

For each species at each site, the total number of individuals collected over the three seasons is indicated. N_{TOTAL} represents the overall individuals collected.

DNA extraction and amplification

Under sterilised conditions, each individual was dissected on ice. The anterior part of the digestive tract, the foregut, and the posterior part, the hindgut, were withdrawn. Therefore, each final sample belonged to four compartments: sediment beneath tentacles, foregut, hindgut and faeces. These microbiomes represent both incoming ASVs of the ingested diet and those that are already present in the digestive tissues. Before extraction, all samples were ground during five minutes, at 30 oscillations per second. DNA was extracted using the DNAeasy 96 PowerSoil Pro kit (Qiagen) following the manufacturer's protocol. The V4–V5 region of 16S rRNA gene was amplified with the primers 515F (GTGCCAGCMGCCGCGGTAA) and 928R (CCCGYCAATTCMTTTRAGT). PCRs were performed with Veriti™ Thermal Cyclers (Applied Biosystems), in a total volume of 20 μ L, with 10 μ L of MasterMix Applied 1X (Applied

Biosystems), 7 µL of milli-Q water, 0.5 µL of forward primer, 0.5 µL of reverse primer and 2 µL of undiluted DNA. The thermocycling program was the following: 94°C for 10 min + 7 × (94°C for 30 s, 62°C [-1°C at each cycle] for 30 s, 72°C for 30 s) + 35 × (94°C for 30 s, 55°C for 30 s, 72°C for 30 s) + 8 × (94°C for 30 s, 56°C for 30 s, 72°C for 30 s) + 72°C for 5 min. The resulting PCR products were sent to the plateforme iGenSeq (ICM, Paris, France) for sequencing with an Illumina MiSeq platform (Illumina, San Diego, CA).

Bioinformatic process

Raw data were analysed with SAMBA v3.0.1 workflow (Standardized and Automatized MetaBarcoding Analysis, <https://github.com/ifremer-bioinformatics/samba>), which is an open-source modular workflow to process eDNA metabarcoding data using DADA2 (Callahan *et al.*, 2016) and QIIME 2 (Bolyen *et al.*, 2019), developed by the SeBiMER (Ifremer's Bioinformatics Core Facility). Raw data integrity was first checked. Then, bioinformatic process was realised with DADA2: (1) sequencing primers were trimmed from reads, (2) bad quality reads were filtered (minQ = 2, FtruncLen = 240 and RtruncLen = 180), (3) sequencing errors were corrected (FmaxEE = 2 and RmaxEE = 2), (4) paired reads have been overlapped, (5) Amplicon Sequence Variants (ASVs) were inferred, and (6) chimeras were removed. Even though DADA2 is a reference tool for bioinformatic process of eDNA metabarcoding data, it induces bias by overestimating diversity due to false-positive ASVs (PCR bias, uncorrected sequencing error). Consequently, dbOTU3 algorithm (Olesen, Duvallet & Alm, 2017) was implemented in SAMBA, which is an additional ASV clustering step, based on the identification of ASVs belonging to the same species and grouping them. dbOTU3 follows three steps: (1) identification of phylogenetically close ASVs, (2) investigation of the distribution of the phylogenetically close ASVs, and (3) analyse of the abundance of the phylogenetically close ASVs. Finally, taxonomy classification was achieved using SILVA database 138 (Quast *et al.*, 2012).

Statistical analyses

All statistical analyses were performed using R (R Core Team, 2023).

The UpSetR plot (similar to a Venn diagram; Conway, Lex & Gehlenborg, 2017; **Figure 22**), established the unique and overlapping ASVs for the four compartments. The nutritional items in the four compartments ('sediment', 'foregut', 'hindgut', 'faeces') are synthesized in **Figure 23**. Combined with the UpSetR plot, which constituted the basis for grouping ASVs, the diagram showed the association of ASVs involved in (1) 'available resources', (2) 'ingestion', (3) 'assimilation', (4) 'excretion', (5) 'foregut bacterial flora', (6) 'hindgut bacterial flora' and (7) 'recolonization of faeces' nutritional items. Working with nutritional items rather than with compartment appears to be more informative, as compartment are involved in several nutritional items. For instance, ASVs from the 'foregut' compartment are involved in three nutritional items, namely, 'foregut bacterial flora', 'ingestion' and 'assimilation'

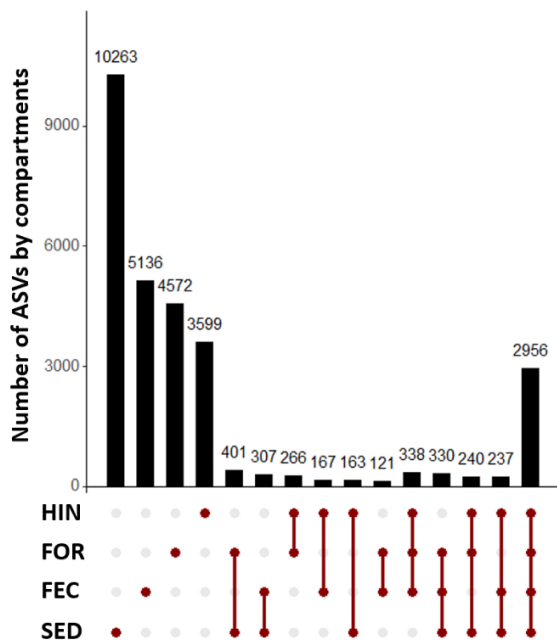


Figure 22: UpSetR visualization of interactions between ASVs within the four compartments. On the left, the four compartments: SED: ‘sediment’; FOR: ‘foregut’; HIN: ‘hindgut’; FEC: ‘faeces’.

The grid below X axis is used to identify the interactions. Unique red coloured dots indicate ASVs found only for one compartment, while connected red coloured dots indicate the number of overlapping ASVs with two or more compartments.

For instance, 4572 ASVs are unique in the ‘foregut’ and 307 ASVs are common to ‘sediment’ and ‘faeces’.

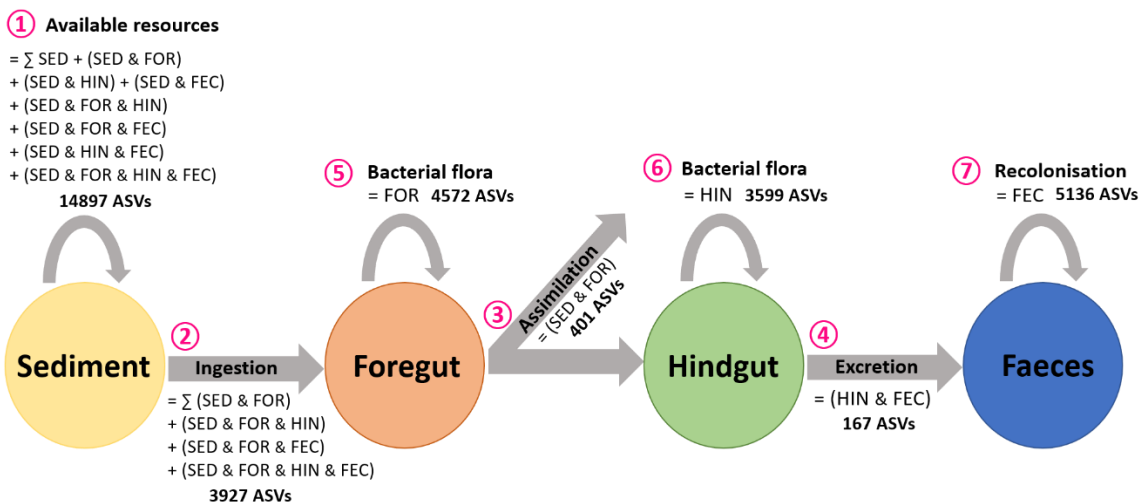


Figure 23: Nutritional items for sea cucumbers, with four external or internal compartments. Combined with the UpsetR plot, this diagram indicates the number of ASVs involved in each of the seven nutritional items highlighted.

The four compartments: SED: ‘sediment’; FOR: ‘foregut’; HIN: ‘hindgut’; FEC: ‘faeces’.

This study focused on the first three nutritional items: 'available resources', 'ingestion' and 'assimilation'. (1) 'Available resources' corresponds to all the ASVs found in the 'sediment' compartment. (2) 'Ingestion' corresponds to ASVs overlapping with at least 'sediment' and 'foregut' compartments, then implying all these combinations: 'sediment' & 'foregut'; 'sediment' & 'foregut' & 'hindgut'; 'sediment' & 'foregut' & 'faeces'; 'sediment' & 'foregut' & 'hindgut' & 'faeces'. The abundance of each ASV from 'foregut', 'hindgut' and 'faeces' were combined, as considered as the real ingested fraction. (3) Finally, the 'assimilation' item corresponds to the ASVs only shared with 'sediment' and 'foregut' compartments. Only the abundance of ASVs within the 'foregut' has been used for the analyses, considered as the real fraction ingested and then assimilated. Finally, the bacteria communities considered here were all benthic, originated from the sediments around the sea cucumbers.

Most of the studies about the nutrition of sea cucumbers based on metabarcoding data have performed analyses at Phylum level. In this study, ASVs were grouped at family level when available. Thus, results mixed several taxa levels for few ASVs, mainly order and family: the denomination used was then 'Order Family'. That represented the best compromise between high taxonomic precision and maximum of ASVs assigned (90% of ASVs were assigned to an 'Order Family' item).

For each nutritional item ('available resources', 'ingestion' and 'assimilation'), alpha diversity with Shannon and Pielou, richness and evenness index respectively, were calculated using the function 'estimate_richness' of the 'phyloseq' R package (McMurdie & Holmes, 2013) for sites, seasons and species. Student *t*-tests were then realised to find out significant differences among sites, seasons and species (function 't_test' of the 'rstatix' R package). A Bonferroni correction was applied.

As different sea cucumber species can ingest and assimilate different fractions of benthic bacteria, and as spatial and temporal variations can occur within species, taxaplots were realised for each species, on each site and for each season, based on one UpSetR plot for each combination. Pairwise ANOSIM were then realised, based on Bray-Curtis distance using the function 'pairwise.adonis' (from the R package 'pairwiseAdonis'; Arbizu, 2019), with

999 permutations and a Bonferroni correction, to compare the benthic bacteria of each nutritional items within seasons at the same site, and within sites for the same species.

Additionally, for each of the three dominants 'Order Family' of each nutritional item, ANOVAs were used to test differences in abundance among species, sites and seasons. Tukey-HSD tests were then applied, when necessary.

Finally, pairwise ANOSIM were realised to compare benthic bacteria from the three nutritional items for each species. SIMPER analyses were then performed, using PAST v4.03 software, to investigate the relative contribution of each 'Order Family' taxa to the significant dissimilarity between nutritional items.

Results

Raw data primary analyses

Sequencing V3–V4 hypervariable region of the 16S rRNA loci resulted in a total of 65,114,161 reads. After quality checking steps, 13,688,380 high-quality reads were selected for analyses, with an average of 7,129 reads per sample. The rarefaction curves of ASVs showed that sequencing depth was sufficient to explain bacterial species richness (**Supplementary material 16**). A total of 29,825 ASVs from 1,919 samples were identified, of which 29,096 were affiliated to the Kingdom of Bacteria and 729 to the Kingdom of Archaea. As marker was used to target Bacteria, the ASVs belonging the Archaea were removed. 1,388 ASVs were not assigned to the Phylum (about 4.5% of ASVs), and 3,282 ASVs were not assigned to the 'Order Family' (about 11% of ASVs). Sequencing revealed 59 Phyla and 613 'Order Family'.

'Available resources': spatio-temporal benthic bacteria profiles in Reunion Island reefs

Globally, the diversity and evenness index of Shannon and Pielou are high, about 3 for Shannon and 0.75 for Pielou (**Figure 24**), meaning that benthic bacteria were highly diversified and that the distribution of benthic bacteria was homogeneous. Concerning the spatial variability, Shannon index was significantly higher at MNS, CAP and PTE than at PLA. The same pattern was observed for Pielou index values, with additionally higher values at ES than at PLA. Consequently, PLA site was characterized by a lower benthic bacteria diversity, more heterogeneous than at other sites. Regarding the temporal variability, benthic bacteria diversity of 2020 Warm season was more diversified and homogeneous than the 2019 Cold season.

For the nutritional item of 'available resources', 15 'Order Family' with relative abundance > 3% were identified, belonging to 8 Phyla (**Figure 25**). The most diversified site was PTE for 2020 Cold season, with the presence of 10 'Order Family'. Three 'Order Family' were dominants and found within all sites and seasons: Pirellulales Pirellulaceae, Chloroplast Chloroplast and Rhodobacterales Rhodobacteraceae, respectively belonging to Planctomycetota, Cyanobacteria and Pseudomonadota Phyla. However, benthic bacteria of all sites were significantly different (**Figure 25; Supplementary material 17**), except for CAP. Additionally, temporal variability was observed (**Figure 25; Supplementary material 18**) with no general trend. In fact, benthic bacteria at MNS and CAP sites was significantly different between the cold and warm seasons. At TE, 2019 Cold and 2020 Warm seasons were different. Finally, at the remaining sites (PAS, PLA, PTE, ES), benthic bacteria structure was significantly different for all seasons. The three dominants 'Order Family' also showed spatio-temporal variability with no general pattern regarding the significant differences (**Supplementary material 19, Supplementary material 20 and Supplementary material 21**).

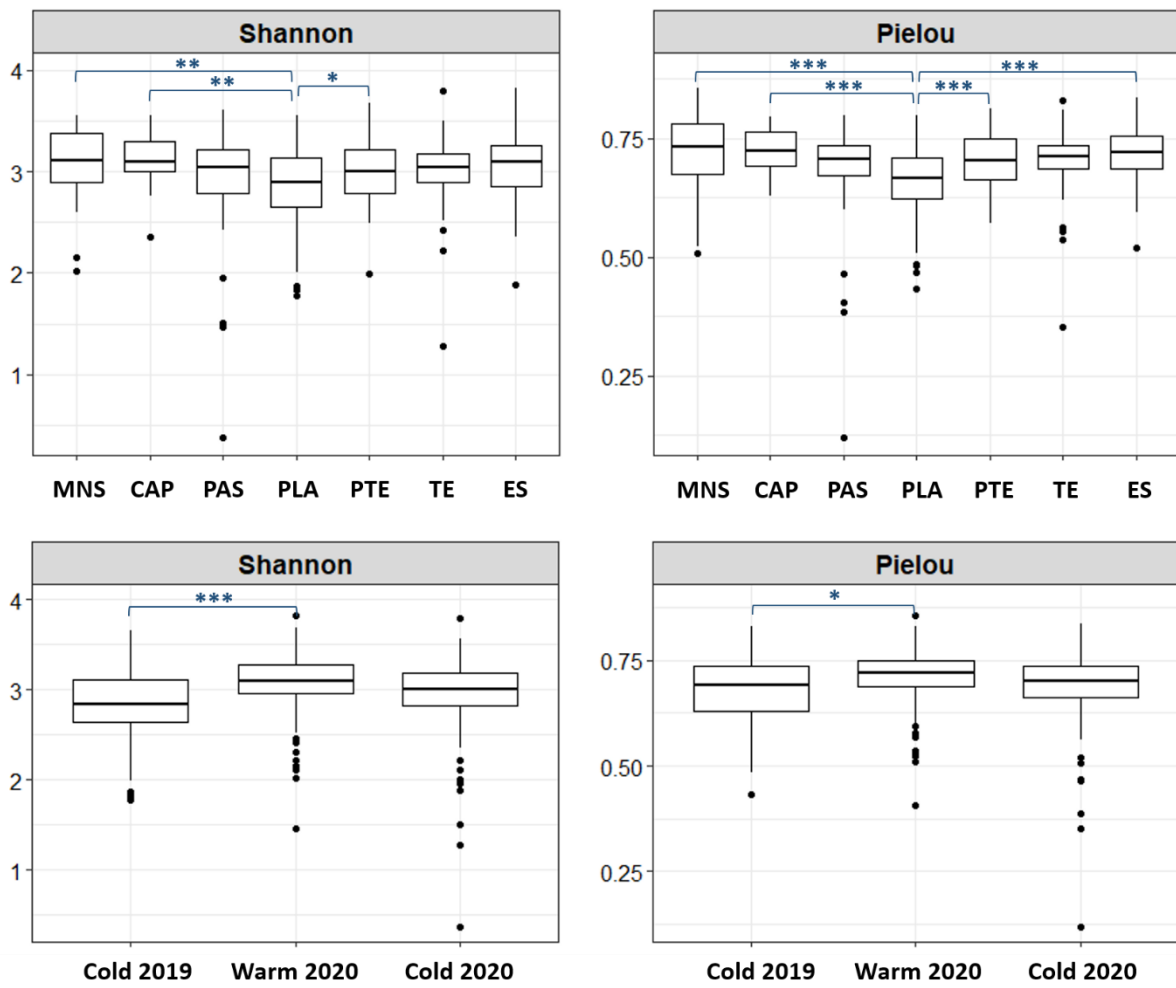


Figure 24: Spatio-temporal variability of the Shannon diversity and Pielou evenness indexes for benthic bacteria included in 'available resources' nutritional item, regarding sites and seasons. Significant differences found with Student t-tests. *P*value are indicated in bold.

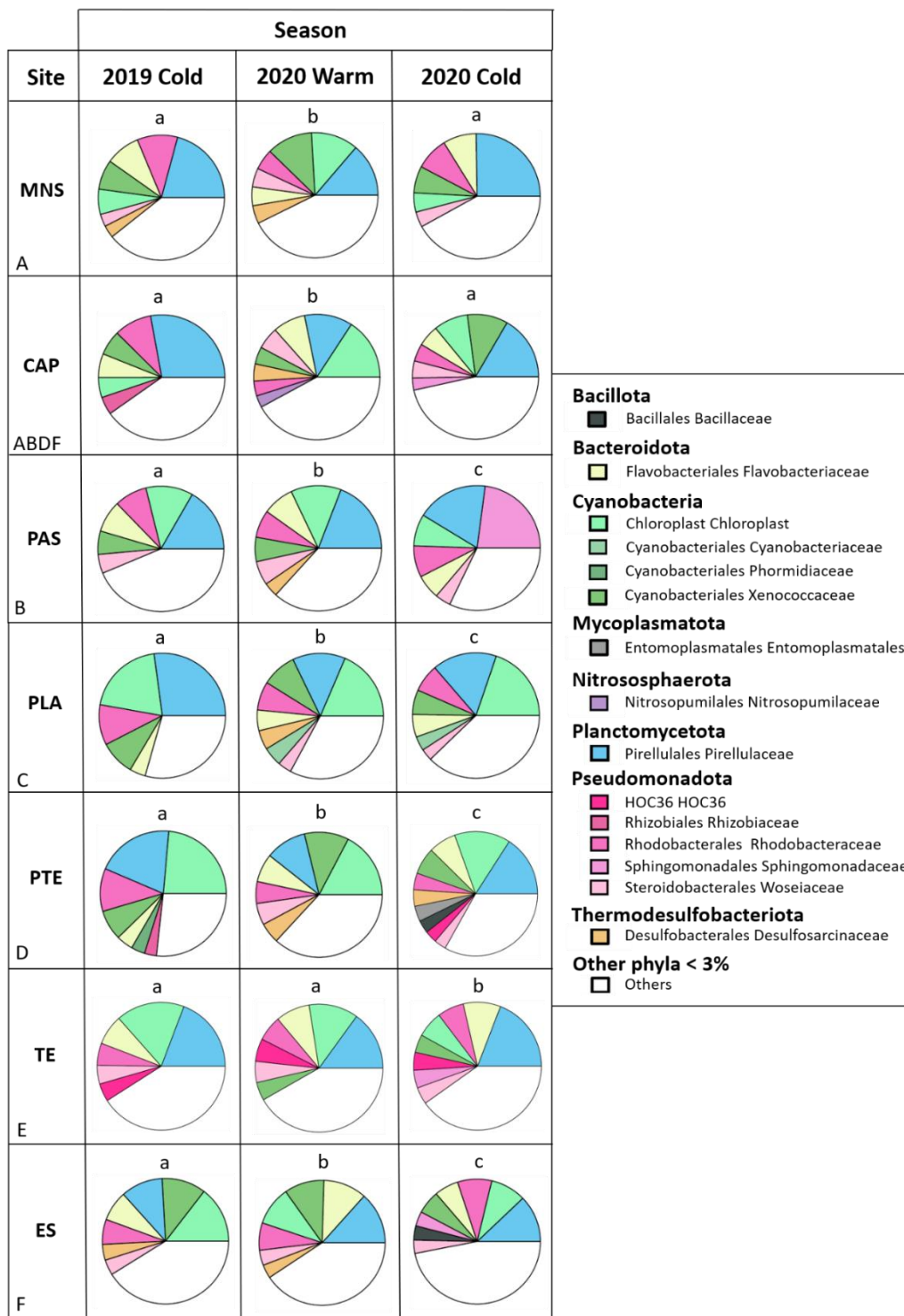


Figure 25: Spatio-temporal diversity of benthic bacteria included in ‘available resources’ nutritional item. Pie charts show the ‘Order Family’ weighing more than 3%, below this threshold all ‘Order Family’ units have been pooled (white caption). Main colour represents ‘Order Family’ from the same Phylum. Different upper cases in the column « Site » indicate significant spatial difference in the diversity of benthic bacteria (ANOSIMs). Different lower cases indicate significant seasonal variation per site (ANOSIMs). Results of ANOSIMs are shown in **Supplementary material 17** and **Supplementary material 18**.

Spatio-temporal variability of benthic bacteria ingested by sea cucumbers

The Shannon index reached about 2.5 (**Figure 26**). Focusing on sea cucumber species, bacteria involved in the 'ingestion' item were significantly the most diversified and homogeneous for *H. leucospilota*. At the intraspecific level, whatever the sea cucumber species, no difference could be found in diversity and homogeneity of bacteria communities across sites. Regarding the season, 2020 Warm season showed higher diversity and evenness than the 2020 Cold season for *H. leucospilota* and *S. chloronotus*. No temporal difference was observed for *H. atra*.

No data were available for MNS site for 2019 Cold season as no faeces have been collected due to rough field conditions. Benthic bacteria involved in the 'ingestion' nutritional item were composed by 25 'Order Family' with relative abundance > 3%, belonging to 9 Phyla (**Figure 27**). The most diversified Phylum was Pseudomonadota with 10 'Order Family'. As for the 'available resources' item, the 'Order Family' Pirellulales Pirellulaceae and Rhodobacterales Rhodobacteraceae were recorded in all species, sites and seasons. Chloroplast Chloroplast was not found for the species *S. chloronotus* at PAS and TE both in 2020 Cold season. Benthic bacteria involved in 'ingestion' item was the most diversified for *H. leucospilota* at CAP for the 2020 Warm season, composed by 11 'Order Family' with relative abundance > 3%. The spatial variability of benthic bacteria of 'available resources' was well reflected at the level of 'ingestion' item, as all holothurian species from different sites ingested significant different benthic bacteria (**Figure 27; Supplementary material 22**). Almost the same temporal pattern as 'available resources' was observed, with the 2020 Warm season significantly different from the two cold seasons at MNS and CAP (**Supplementary material 23**), as well as for PTE. Benthic bacteria involved in the 'ingestion' item were significantly different for all seasons at PAS, PLA, TE and ES. The three dominant 'Order Family', that were Pirellulales Pirellulaceae, Rhodobacterales Rhodobacteraceae and Sphingomonadales Sphingomonadaceae, exhibited specific, spatial and seasonal significant differences

(**Supplementary material 24, Supplementary material 25, Supplementary material 26**), and no global pattern could be evidenced.

Diversity in benthic bacteria from ‘available resources’ and ‘ingestion’ item were significantly different for *S. chloronotus* (pairwise-ANOSIM, $R= 0.36$, $Pvalue < 0.001$), *H. atra* (pairwise-ANOSIM, $R= 0.37$, $Pvalue < 0.001$) and *H. leucospilota* (pairwise-ANOSIM, $R= 0.28$, $Pvalue < 0.001$). Interestingly, bacteria found in ‘ingestion’ showed low variability between holothurian species (*H. leucospilota* and *H. atra*, $R= 0.051$; *H. leucospilota* and *S. chloronotus*, $R= 0.107$; *H. atra* and *S. chloronotus*, $R= 0.074$; $Pvalues < 0.01$). SIMPER analyses revealed that Pirellulales Pirellulaceae had a high contribution (about 30%) to the dissimilarity between those two nutritional items for *S. chloronotus* at all sites and seasons (**Supplementary material 27**), followed by Rhodobacterales Rhodobacteraceae (15%), with a mean abundance of 711 (± 216) and 324 (± 81) ASVs for ‘available resources’ and 6813 (± 1420) and 3190 (± 666) ASVs for ‘ingestion’ item, respectively. Pirellulales Pirellulaceae was also the ‘Order Family’ which contributed the most to the dissimilarity between the two nutritional items for both *H. atra* (21%, **Supplementary material 28**) and *H. leucospilota* (18%, **Supplementary material 29**): with respectively, means of 1513 (± 654) and 888 (± 434) ASVs for ‘available resources’ and 5880 (± 1100) and 3823 (± 1242) for ‘ingestion’ item. Finally, the number of taxa necessary to reach 50% of dissimilarity was lower for *S. chloronotus* (mean of 2.7 ‘Order Family’), than for *H. atra* (3.7), than for *H. leucospilota* (4.9).

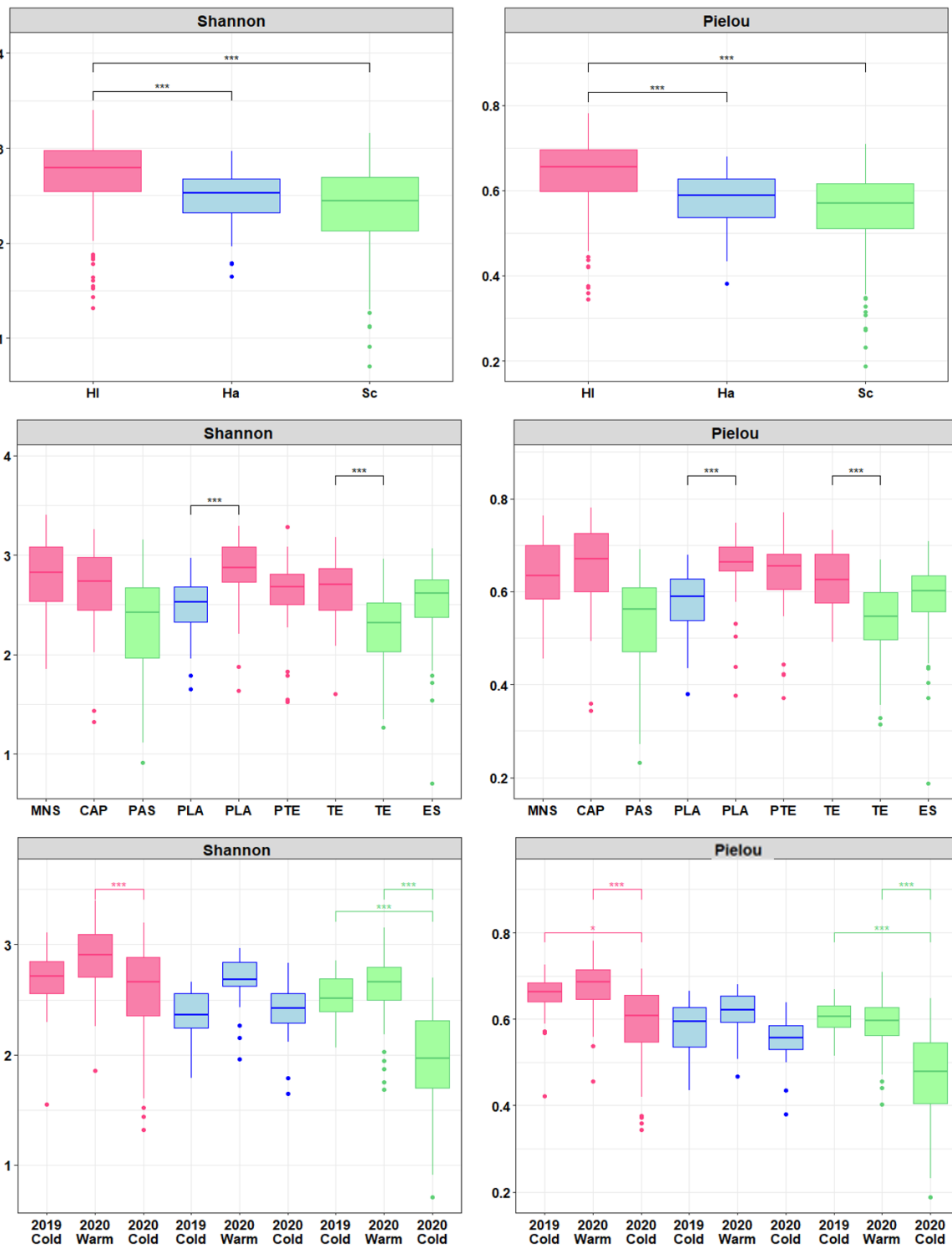


Figure 26: Spatio-temporal and species variability of the Shannon diversity and Pielou evenness indexes of benthic bacteria in ‘ingestion’ nutritional item. Significant differences found with Student *t*-tests are indicated: *P*value < 0.05: *; < 0.01: ** and < 0.001: ***. Only significant values among the same species are represented (intraspecific), and between species from the same site (intra site; PLA and TE). *Holothuria leucospilota* in pink; *Holothuria atra* in blue and *Stichopus chloronotus* in green.

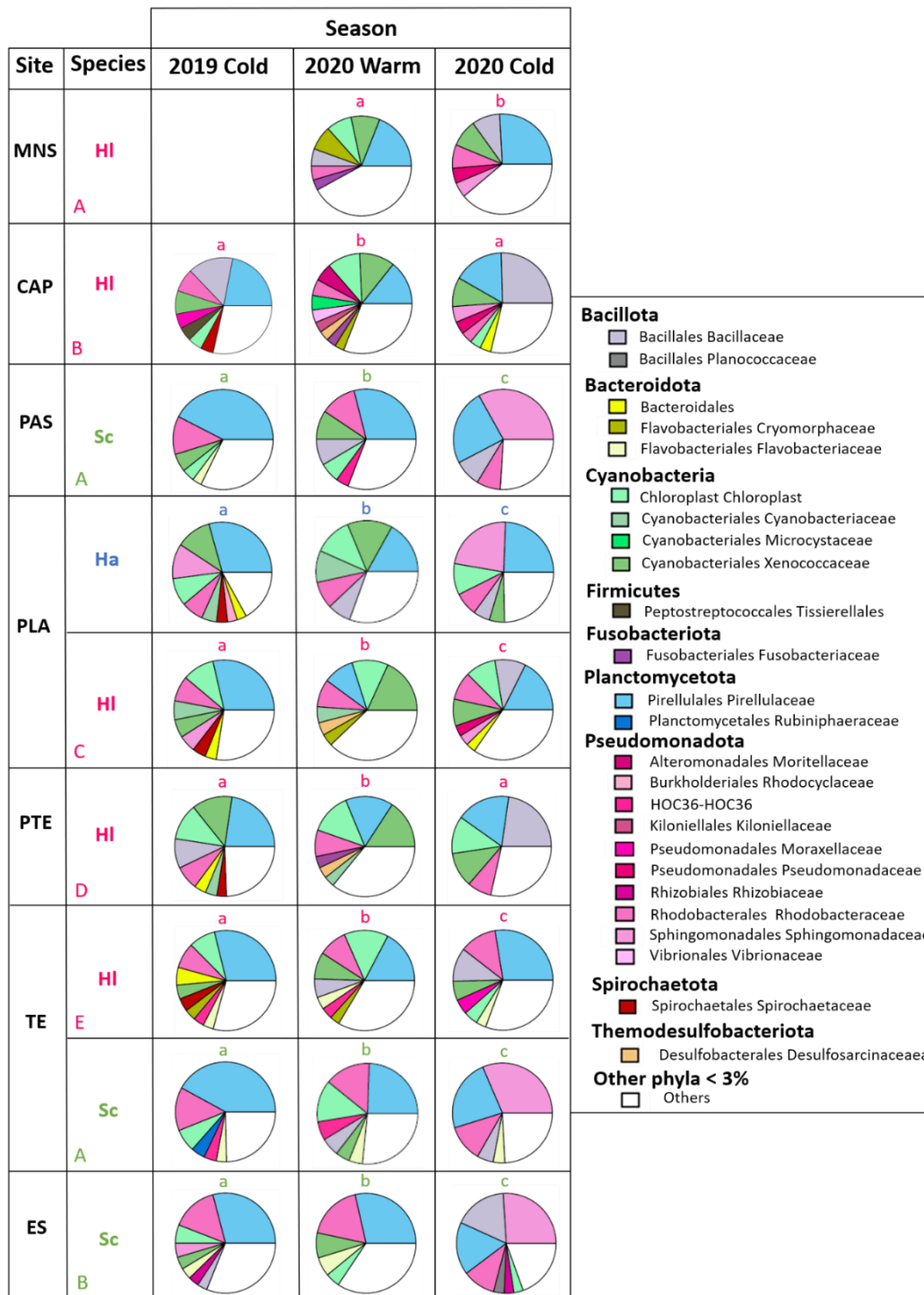


Figure 27: Spatio-temporal diversity of benthic bacteria involved in the ‘ingestion’ nutritional item for each species. Pie charts show the ‘Order Family’ weighing more than 3%, below this threshold all ‘Order Family’ units have been pooled (white caption). Main colour represents ‘Order Family’ from the same Phylum. Different upper cases of the same colour in the « Species » column indicate significant spatial difference in the diversity of benthic bacteria for each species (ANOSIMs). For each row, different lower cases indicate significant seasonal variation per site and species (ANOSIMs). Results of ANOSIMs are shown in **Supplementary material 22** and **Supplementary material 23**.

Spatio-temporal variability of benthic bacteria assimilated by sea cucumbers

Shannon diversity index was low about 1.5, and the Pielou evenness index was about 0.65 (**Figure 28; Supplementary material 28**) for 'Assimilation' item. Bacteria involved in 'assimilation' item were more diversified for *H. leucospilota* than the two other species. Consequently, significant difference was found for the *H. leucospilota* and *H. atra* at PLA. At sea cucumber intraspecific level, bacteria in 'assimilation' item followed the same pattern as the 'ingestion' item, with no significant difference. Shannon index showed dissimilarity for 2019 and 2020 Cold seasons for *S. chloronotus*, while Pielou index was different between 2020 Warm and Cold seasons for *H. leucospilota*.

The 'assimilation' item was featured by 22 'Order Family', belonging to 8 Phyla and including 4 'Order Family' not recorded in the 'ingestion' item at more than 3%. The latter were Clostridiales Clostridiaceae, Cytophagales Cyclobacteriaceae belonging respectively to the Bacillota and Bacteroidota Phyla, and NB1-j NB1-j and Steroidobacterales Woeseiaceae, both belonging to the Pseudomonadota Phylum (**Figure 29**). For this analysis only, the relative abundance of the 'Not assigned' ASVs was higher than 3%. None of the 'Order Family' taxa identified was shared by all species or sites or seasons, conversely to the 'available resources' and the 'ingestion' items. Sea cucumber species seemed to assimilate different fractions of benthic bacteria as a high relative abundance of Bacteroidota and Spirochaetales Spirochaetaceae was observed for *H. leucospilota*, and of Pirellulales Pirellulaceae and Rhodobacterales Rhodobacteraceae for *S. chloronotus*. Additionally, the number of the 'Order Family' involved in the 'assimilation' item of each species was different, with an average of 4.4 'Order Family' for *S. chloronotus*, 4.6 for *H. atra* and 7.3 for *H. leucospilota*. Benthic bacteria involved in the 'assimilation' item displayed no particular spatial and temporal pattern (**Figure 29, Supplementary material 29; Supplementary material 30 and Supplementary material 31**). Similar results were observed when selecting the three dominant 'Order Family' only, Pirellulales Pirellulaceae, Rhodobacterales Rhodobacteraceae and Sphingomonadales Sphingomonadaceae (**Supplementary material 32; Supplementary material 33 and Supplementary material 34**).

Diversity in benthic bacteria involved in the 'ingestion' and 'assimilation' nutritional items was significantly different, for *S. chloronotus* (pairwise-ANOSIM, $R= 0.20$, P value < 0.001), *H. atra* (pairwise-ANOSIM, $R= 0.30$, P value < 0.001) and *H. leucospilota* (pairwise-ANOSIM, $R= 0.23$, P value < 0.001). As for the 'ingestion' item, benthic bacteria involved in the 'assimilation' item were mildly different between species (*H. leucospilota* and *H. atra*, $R= 0.066$; *H. leucospilota* and *S. chloronotus*, $R= 0.155$; *H. atra* and *S. chloronotus*, $R= 0.037$; P values < 0.01). *Pirellulales* *Pirellulaceae* were preferentially ingested by *S. chloronotus*, contributing to 23% dissimilarity between the two nutritional items, with 6813 ($\pm 1,420$) and 2581 (± 651) ASVs involved in the 'ingestion' and 'assimilation' items respectively (**Supplementary material 35**). Some other 'Order Family' were recorded in 'ingestion' item by *S. chloronotus*, but were not found in 'assimilation' item at some sites and seasons, like *Cyanobacteriales* *Xenococcaceae*, *Sphingomonadales* *Sphingomonadaceae* and *Bacillales* *Bacillaceae*. The same pattern was observed for *H. atra* and *H. leucospilota*, with *Pirellulales* *Pirellulaceae* contributing the most to the dissimilarity between nutritional items (20% for both species): with 5880 (± 1100) and 3823 (± 477) ASVs for 'ingestion' and 1800 (± 325) and ASVs for 'assimilation' item, respectively (**Supplementary material 36 and Supplementary material 37**). Similarly, for *H. atra* and *H. leucospilota*, some 'Order Family' were found in 'ingestion' item but were not observed in 'assimilation' item, as *Chloroplast Chloroplast* or *Cyanobacteriales* *Xenococcaceae* for *H. atra* and *Pirellulales* *Pirellulaceae*, *Bacillales* *Bacillaceae* or *Cyanobacteriales* *Xenococcaceae* for *H. leucospilota*. The number of 'Order Family' involved in 50% of the dissimilarity between the two nutritional items was quite similar for *S. chloronotus* (3.3) and *H. atra* (3.6), but higher for *H. leucospilota* (4.4).

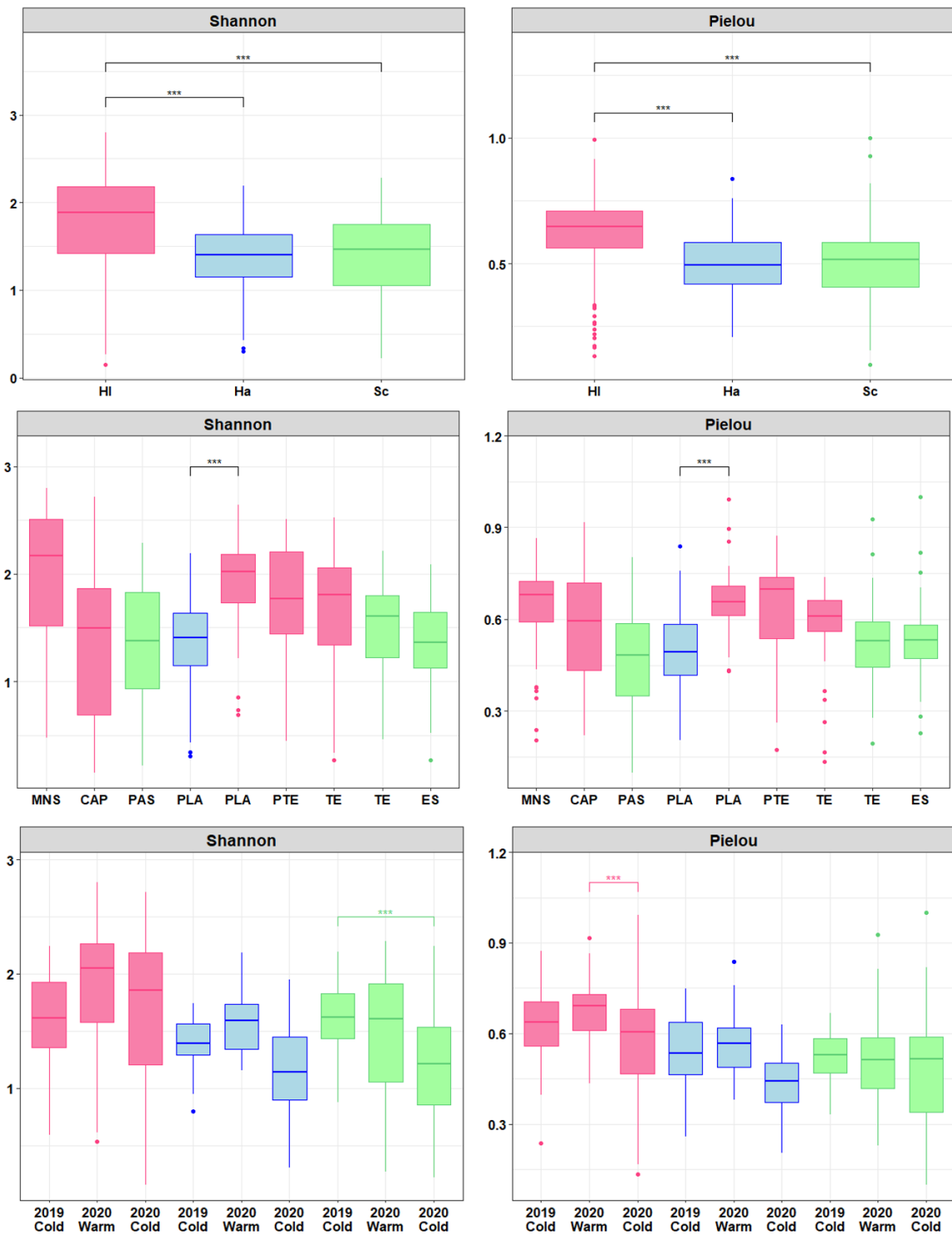


Figure 28: Spatio-temporal and species variability of the Shannon diversity and Pielou evenness indexes for benthic bacteria involved in ‘assimilation’ nutritional item, at all sites and seasons. Significant differences found with Student *t*-tests are indicated: *P*value < 0.05: *; < 0.01: ** and < 0.001: ***. Only significant values among the same species are represented, and between species from the same site (PLA and TE). *Holothuria leucospilota* in pink; *Holothuria atra* in blue and *Stichopus chloronotus* in green.

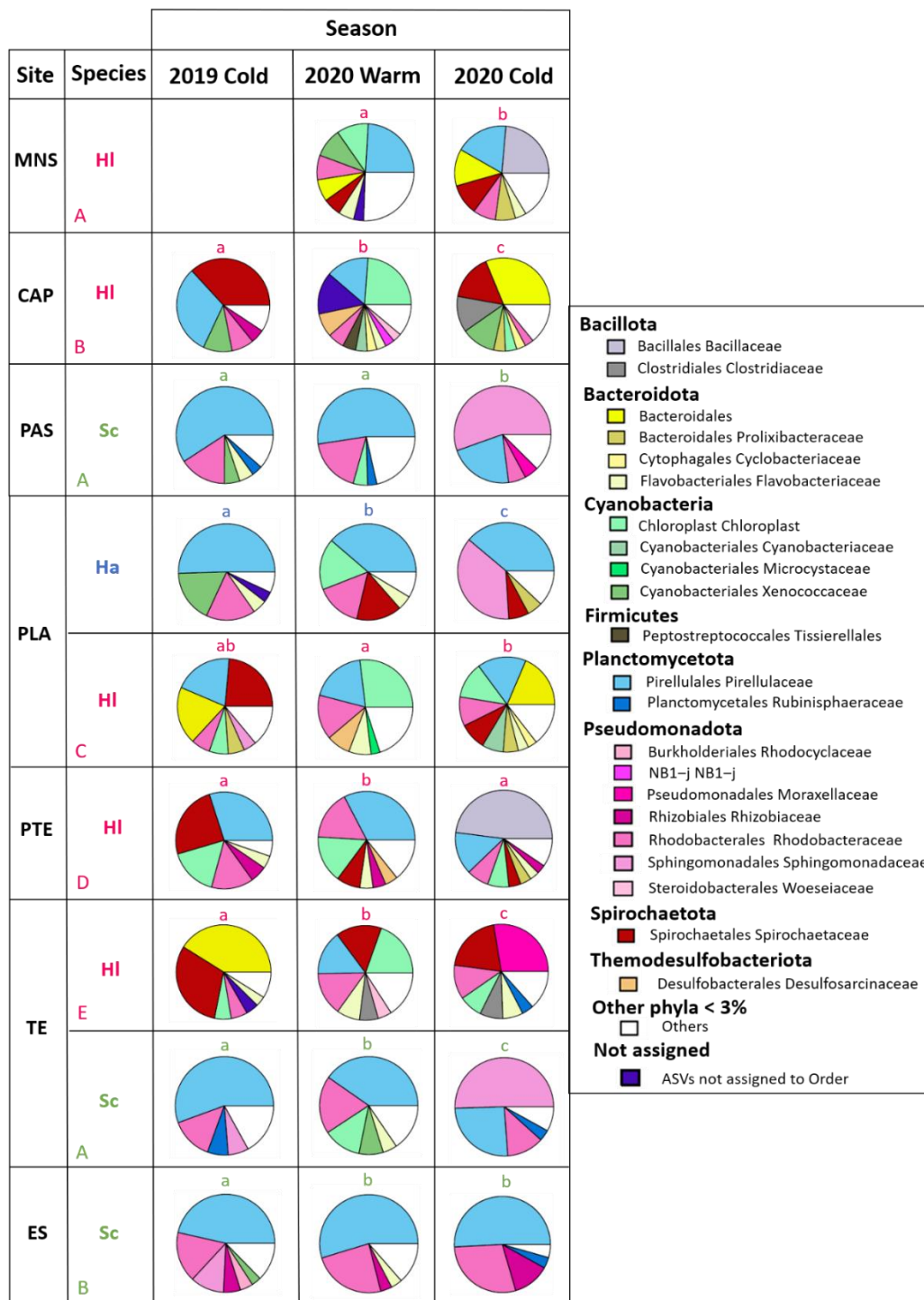


Figure 29: Spatio-temporal diversity of benthic bacteria involved in the ‘assimilation’ nutritional item for each species. Pie charts show the ‘Order Family’ weighing more than 3%, below this threshold all ‘Order Family’ units have been pooled (white caption). Main colour represents ‘Order Family’ belonging to the same Phylum. Different upper cases of the same colour in the « Species » column indicate significant spatial difference in the diversity of benthic bacteria for each species (ANOSIMs). For each row, different lower cases indicate significant seasonal variation per site and species (ANOSIMs). Results of ANOSIMs are shown **Supplementary Materials 30 and 31.**

Discussion

Spatio-temporal variability of benthic bacteria in Reunion Island reefs and relation to the patchy distribution of sea cucumbers

Bacteria associated to substrates within the two coral reefs in this study presented strong spatial and temporal variability. The main 'Order Family' taxa associated with substrates were Pirellulales Pirellulaceae, Rhodobacterales Rhodobacteraceae, Chloroplast Chloroplast and Flavobacteriales Flavobacteriaceae, respectively belonging to Planctomycetota, Pseudomonadota (previously named as Proteobacteria), Cyanobacteria and Bacteroidota. These Phyla have already been reported in coral reef benthic compartments. Dong *et al.* (2022) highlighted that Pseudomonadota, with similar Orders (Rhodobacterales, Shingomonadales, or Rhizobiales) and Planctomycetota (mainly Pirellulales) were the main benthic bacteria Phyla for 16 coral reefs in Xisha Island. Coral reef benthic bacteria of Hawaii were mainly dominated by Planctomycetota, Pseudomonadota, and Bacteroidota (Rusch, Hannides & Gaidos, 2009). Finally, Pseudomonadota largely dominated benthic bacteria of Jakarta reefs, with a very low proportion of Cyanobacteria (De Voogd *et al.*, 2015). Benthic bacteria diversity at higher taxa seems therefore similar within tropical coral reefs at large-scale, with the same main dominating Phyla.

Strong spatial and temporal difference of benthic bacteria were observed with no general trend. In paralleled, sea cucumber species of Reunion Island presented a patchy distribution with areas of high density and low density. Therefore, the patchy distribution observed for sea cucumbers seems not related to the highly variable distribution of benthic bacteria. No study had highlighted such correlation between bacteria and sea cucumbers, either for other echinoderm taxa. The patchy distribution of sea cucumbers of Reunion Island could be link to others food resources, such as microalgae (Uthicke & Karez, 1999).

The presence of Cyanobacteria is related to the eutrophication of waters by anthropogenic pollutants (Touchette *et al.*, 2007; Lürling, Van Oosterhout & Faassen, 2017). Cyanobacteria were found at all sites and all seasons, supporting the eutrophicated status of Reunion Island coral reefs.

Implication of benthic bacteria in the nutritional items of sea cucumbers: does any selection occur?

No study has investigated the implication of benthic bacteria in nutritional items of sea cucumbers, as they focused mainly on the sediment and gut compartments, with no understanding about the ingestion and assimilation or any other nutritional item. The most studied species is the farmed *Apostichopus japonicus*. Gao *et al.* (2014) evidenced that the main Phylum in the foregut and hindgut were Pseudomonadota, with a dominance of γ -proteobacteria. Planctomycetota were also mainly recorded in the foregut. Wang *et al.* (2018) showed that Pseudomonadota largely dominated the foregut (relative abundance > 80%). Benthic bacteria were very similar between the midgut and the hindgut, with mainly Pseudomonadota (about 20%) and Bacillota (previously Firmicutes, about 20%). Bacteroidota and Cyanobacteria were also found in lower proportions. Sha *et al.* (2016) observed the same Phyla in the gut of *A. japonicus*, with Pseudomonadota dominating (> 50%), as well as Pagán-Jiménez *et al.* (2019) in the foregut of *Holothuria glaberrima*. Plotieau *et al.* (2013) examined benthic bacteria diversity in different parts of the gut of *Holothuria scabra*. They highlighted that the main Phyla were Pseudomonadota (with a dominance of γ -proteobacteria), Bacteroidota, Bacillota, and a lower relative abundance for Cyanobacteria (< 2%). This is similar to results from our study, except for Cyanobacteria, what may be due to the use of individuals from aquaculture conditions in other studies, while this study has focused on wild individuals.

The previous studies evidence that benthic bacteria diversity was different between the sediment and the foregut, similarly to this study where dissimilarity between the 'available resources' and 'ingestion' item was demonstrated. Plotieau *et al.* (2013) and Gao *et al.* (2014) hypothesized that individuals select bacteria before ingestion. Ciliated cells in the bud epidermis of tentacles seem to be sensory (Bouland, Massin & Jangoux, 1982; Flammang & Conand, 2004) and could therefore be involved in the selection of bacteria. The dissimilarity observed between benthic bacteria in the sediment and in the foregut could also be linked to the vertical distribution of benthic bacteria. Rusch *et al.* (2009) showed that benthic bacteria richness was different between oxic (up to 15 cm) and interfacial (up to 25 cm) sediments.

Hewson & Fuhrman (2006) found dissimilarity in benthic bacteria richness for sediment between 0 to 3 cm and 3 to 5 cm. The foraging depth by *H. atra* can reach 4 cm, while these by *H. leucospilota* and *S. chloronotus* are superficial, down to 1 cm (Taddei, 2006). Consequently, the foraging depth of these three sea cucumbers species can explain in part the dissimilarity observed between benthic bacteria diversity from sediment and foregut. *Holothuria leucospilota* presented the high diversity and evenness of benthic bacteria compared to the two other species. This could be link to the huge amount of sediment processed, about 100 g.ind⁻¹.d⁻¹ (Mangion *et al.*, 2004), comparatively to *S. chloronotus*, about 6 g.ind⁻¹.d⁻¹, and *H. atra* 30 g.ind⁻¹.d⁻¹ (Klinger, Johnson & Jell, 1994). Consequently, *H. leucospilota* appears to be more 'efficient sampler'. Finally, Pierrat *et al.* (2022) clarified the selection process of sea cucumbers, which tend to select fine organically rich particles. This type of particles hosts higher bacteria biomass due to the higher surface-to-volume ratio (Johnstone, Koop & Larkum, 1990). In consequence, further studies should focus on the richness of bacteria on organically rich particles, which can be peculiar, and can explain the dissimilarity observed between benthic bacteria in sediment and foregut compartments for sea cucumbers. Our results concerning the spatio-temporal and specific variability of benthic bacteria at medium taxonomic rank (Order Family) indicated that each sea cucumber population within the reefs of L'Hermitage/La Saline et Étang-Salé ingested and assimilated different bacterial communities with no global pattern. Even if sea cucumbers are able to select bacteria, it seems the individuals in Reunion ingested random fractions of benthic bacteria present in sediment, meaning that no selection of some peculiar bacterial taxa occurred.

Almost 16% of the benthic bacteria identified were only found in the foregut and 12% in the hindgut. Some shared benthic bacteria have been found anywhere else than in the foregut and the hindgut: these count about 1% of the total identified ASVs. These ASVs have not been found in other compartments due to their absence or maybe to some detection limitation. Such ASVs could evidence some endogenous bacteria present in the foregut and the hindgut. Similarly, about another 1% of the ASVs were overlapping from foregut, hindgut and faeces. None of those ASVs being present in sediments, it could be inferred that some endosymbiotic bacterial production could occur in the assimilation process. These results

could potentially evidence intestinal microflora of sea cucumbers, already reported by few studies (Deming & Colwell, 1982; Harris, 1993), but with limited technics. The bacteria could help sea cucumbers during the digestive processes (Deming & Colwell, 1982; Harris, 1993). Recently, Quintanilla-Mena *et al.* (2022) have identified some strains in the gut of *Isostichopus badionotus* with probiotic and bioremediation potentials, namely *Acinetobacter*, *Lactobacillus*, *Enterobacter*, *Enterococcus* or *Shewanella*. Authors hypothesized that these strains are involved in metabolism, genetic and environmental information processing, organismal systems and cellular processes. Yet, the hypothesis of bacterial sources other than sediments has to be considered, especially for *H. leucospilota* or *S. chloronotus*, which are epibenthic deposit-feeders, feeding on various substrates (Hammond & Wilkinson, 1985; Roberts *et al.*, 2000; Cuvillier, 2016).

Whole snapshot of the diet of sea cucumbers: other resources involved

This study assessed only one fraction of the diet of sea cucumbers, the benthic bacteria. However, diet of sea cucumbers is more diversified and complex. In fact, the living fraction also includes photosynthetic organisms and meiofauna. Some recent studies have focused on the eukaryotes involved in the diet of sea cucumbers. Dinoflagellata, Mollusca and Bacillariophyceae were dominant in the gut of the farmed *A. japonicus* (Zhang *et al.*, 2016). The eukaryote relative abundance was heterogenous for some wild species of sea cucumbers, with Diatomea and Arthropoda, being the main Phyla recorded in the gut of *Stichopus monotuberculatus*, while Apicomplexa, Diatomea and non-assigned Eukaryote dominated in the gut of *S. chloronotus* and *H. atra* (Jia *et al.*, 2022). The eukaryote relative abundance found in the gut of these three species showed strong dissimilarity with the main Phyla recorded in sediments, Annelida and Nematoda (Jia *et al.*, 2022). Finally, Zhang *et al.* (2023) examined the eukaryote diversity in different parts of the gut of *H. leucospilota*. In the foregut, Nematoda, Mollusca and Chlorophyta (each > 10%) were the most abundant Phyla identified, while Streptophyta largely dominated in the hindgut (> 45%). Authors stated that selection occurs as the main Phyla found in the sediment were Annelida and Arthropoda (each > 20%). In conclusion, the eukaryote fraction of the diet of sea cucumber seem to vary among species. In consequence, metabarcoding analyses with combination of several gene markers, as 16S

Bacteria and Archaea, 18S Eukaryota, should be conducted over several seasons and locations to fully understand the diet of sea cucumbers.

Additionally, other reef compartments should be investigated. A recent study of the bacterial diversity of the reef waters of Reunion Island highlighted that Pseudomonadota, Cyanobacteria and Actinomycetota were the main dominant Phyla (Rapport BIOINDIC, Jourand, 2022). Rhodobacteraceae was the main Family recorded for Pseudomonadota (Rapport BIOINDIC, Jourand, 2022), as found in this study. Epibenthic deposit-feeding sea cucumbers also forage in other compartments than sediment, as for instance seagrasses, tegument of other specimens (J.P., personal observations), boulders, rubbles and corals (Roberts *et al.*, 2000), that have not been considered in this study nor in the literature. Such substrata could be characterized by specific bacterial or eukaryotic diversity and should be considered for complete snapshot of sea cucumbers diet.

Conclusion

This study showed that benthic bacteria of Reunion Island are highly diversified and mainly dominated by Pirellulales Pirellulaceae, Rhodobacterales Rhodobacteraceae and Chloroplast Chloroplast Cyanobacteria, with strong seasonal and spatial variability. Consequently, benthic bacteria cannot be considered as a key factor explaining the patchy high-density distribution observed of sea cucumber populations at Reunion Island. The bacterial features for the three nutritional items 'available resources', 'ingestion' and 'assimilation' were not similar. The three species ingested and assimilated almost the same fraction of benthic bacteria, mostly Pirellulales, especially Shingomonadales and Spirochaetales for *H. atra*, Spirochaetales and Chloroplast for *H. leucospilota*, and Shingomonadales and Rhodobacterales for *S. chloronotus*. Cyanobacteria were recorded at all sites and seasons, possibly resulting from the eutrophication of the reefs at Reunion Island. Further studies should investigate the compartments as water column, hard substrata, macroalgae or seagrasses as sources of bacteria. Other gene markers such as Archaea and Eukaryotes should also be investigated to get a complete snapshot of the diet of sea cucumbers.

Acknowledgment

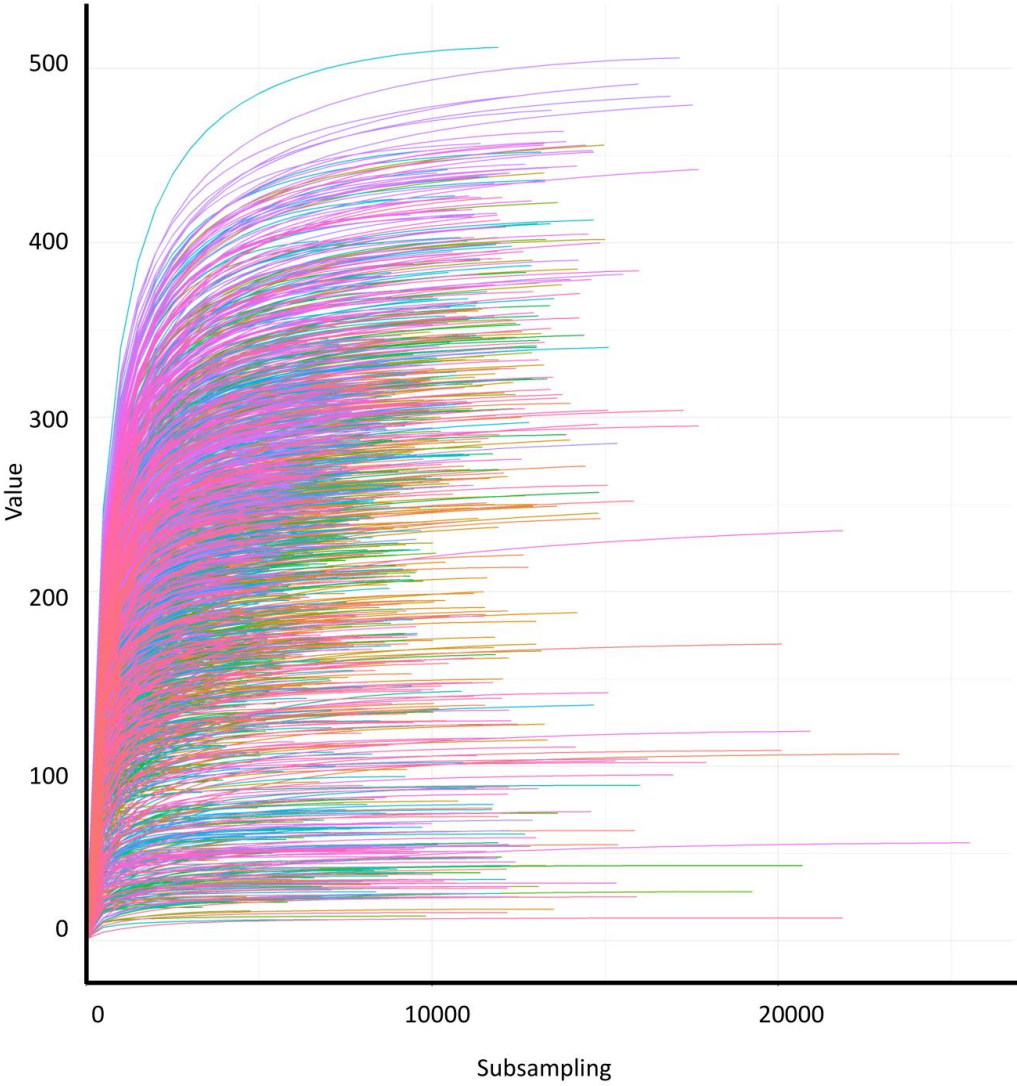
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Supplementary Material

Supplementary material 16: Rarefaction curves showing the relation between the number of reads and ASV numbers.



Supplementary material 17: Benthic bacteria inter-site variability. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold.

Station	ANOSIM	R= 0.117		Pval < 0.001		
	MNS	CAP	PAS	PLA	PTE	TE
CAP	> 0.05	–				
PAS	0.021	> 0.05	–			
PLA	0.021	0.021	0.021	–		
PTE	0.021	> 0.05	0.021	0.021	–	
TE	0.021	0.021	0.021	0.021	0.021	–
ES	0.021	> 0.05	0.021	0.021	0.021	0.021

Supplementary material 18: Benthic bacteria intra-site seasonal variability. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold.

MNS	ANOSIM	<i>R</i> = 0.199	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.009	–	
2020 Cold	> 0.05	0.003	–
CAP	ANOSIM	<i>R</i> = 0.198	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	> 0.05	0.03	–
PAS	ANOSIM	<i>R</i> = 0.144	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.009	–	
2020 Cold	0.003	0.003	–
PLA	ANOSIM	<i>R</i> = 0.132	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.003	0.003	–
PTE	ANOSIM	<i>R</i> = 0.185	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.018	0.027	–
TE	ANOSIM	<i>R</i> = 0.070	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	> 0.05	–	
2020 Cold	0.003	0.003	–
ES	ANOSIM	<i>R</i> = 0.119	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.03	0.003	–

Supplementary material 19: Inter-site and inter-seasonal variability of benthic bacteria *Pirellulales Pirellulaceae* in the 'available resources' nutritional item. Results of ANOVA and Tukey HSD. Significant Pvalue are indicated in bold

Available resources: <i>Pirellulales Pirellulaceae</i>						
Site	ANOVA	F= 5.221	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	> 0.05	> 0.05	–			
PLA	0.04	> 0.05	> 0.05	–		
PTE	> 0.05	> 0.05	> 0.05	0.02	–	
TE	> 0.05	> 0.05	> 0.05	0.002	> 0.05	–
ES	> 0.05	> 0.05	> 0.05	< 0.001	> 0.05	> 0.05
Season	ANOVA	F= 9.287	Pval < 0.001			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	< 0.001	–				
Cold 2020	> 0.05	0.02	–			

Supplementary material 20: Inter-site and inter-seasonal variability of benthic bacteria Chloroplast Chloroplast in the 'available resources' nutritional item. Results of ANOVA and Tukey HSD. Significant Pvalue are indicated in bold.

Available resources: Chloroplast Chloroplast						
Site	ANOVA	F= 41.77	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	> 0.05	> 0.05	–			
PLA	< 0.001	< 0.001	< 0.001	–		
PTE	< 0.001	0.003	> 0.05	< 0.001	–	
TE	> 0.05	> 0.05	> 0.05	< 0.001	0.007	–
ES	> 0.05	> 0.05	> 0.05	< 0.001	0.03	> 0.05
Season	ANOVA	F= 4.764	Pval = 0.008			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	> 0.05	–				
Cold 2020	0.008	> 0.05	–			

Supplementary material 21: Inter-site and inter-seasonal variability of benthic bacteria Rhodobacterales Rhodobacteraceae in the 'available resources' nutritional item. Results of ANOVA and Tukey HSD. Significant Pvalue are indicated in bold.

Available resources: Rhodobacterales Rhodobacteraceae						
Site	ANOVA	F= 7.273	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	> 0.05	> 0.05	–			
PLA	< 0.001	< 0.001	> 0.05	–		
PTE	> 0.05	> 0.05	> 0.05	0.02	–	
TE	> 0.05	> 0.05	> 0.05	< 0.001	> 0.05	–
ES	> 0.05	> 0.05	> 0.05	0.03	> 0.05	
Season	ANOVA	F= 4.243	Pval < 0.001			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	0.01	–				
Cold 2020	> 0.05	> 0.05	–			

Supplementary material 22: Inter-site variability of benthic bacteria involved in the ‘ingestion’ nutritional item. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold.

HI	ANOSIM	<i>R</i> = 0.106	<i>P</i> val < 0.001		
Station	MNS	CAP	PLA	PTE	TE
MNS	–				
CAP	0.02	–			
PLA	0.01	0.01	–		
PTE	0.01	0.04	0.01	–	
TE	0.01	0.01	0.01	0.01	–
Sc	ANOSIM	<i>R</i> = 0.041	<i>P</i> val < 0.001		
Station	PAS	TE	ES		
PAS	–				
TE	0.057	–			
ES	0.015	0.003	–		

Supplementary material 23: Intra-site seasonal variability of benthic bacteria involved in the ‘ingestion’ nutritional item. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold.

MNS HI	ANOSIM	<i>R</i> = 0.188	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.552	0.003	–
PAS Sc	ANOSIM	<i>R</i> = 0.303	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.003	0.003	–
PLA Ha	ANOSIM	<i>R</i> = 0.295	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.003	0.003	–
PLA HI	ANOSIM	<i>R</i> = 0.261	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.003	0.003	–
PTE HI	ANOSIM	<i>R</i> = 0.180	<i>P</i> val = 0.002
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.027	–	
2020 Cold	0.369	0.003	–
TE HI	ANOSIM	<i>R</i> = 0.106	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.012	–	
2020 Cold	0.012	0.006	–
TE HI	ANOSIM	<i>R</i> = 0.293	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.009	–	
2020 Cold	0.003	0.003	–
ES Sc	ANOSIM	<i>R</i> = 0.224	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.006	–	
2020 Cold	0.006	0.003	–

Supplementary material 24: Inter-site and inter-seasonal variability of benthic bacteria *Pirellulales Pirellulaceae* in the ‘ingestion’ nutritional item.

Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold.

Ingestion: Pirellulales Pirellulaceae						
Site	ANOVA	F= 13.62	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	< 0.001	< 0.001	–			
PLA	> 0.05	> 0.05	< 0.001	–		
PTE	> 0.05	> 0.05	< 0.001	> 0.05	–	
TE	> 0.05	0.006	< 0.001	> 0.05	0.01	–
ES	0.03	0.002	0.01	> 0.05	0.005	> 0.05
Season	ANOVA	F= 11.12	Pval < 0.001			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	< 0.001	–				
Cold 2020	> 0.05	0.009	–			
Specie	ANOVA	F= 51.94	Pval < 0.001			
Season	Ha	HI	Sc			
Ha	–					
HI	< 0.001	–				
Sc	> 0.05	< 0.001	–			

Supplementary material 25: Inter-site and inter-seasonal variability of benthic bacteria Rhodobacterales Rhodobacteraceae in the ‘ingestion’ nutritional item. Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold.

Ingestion: Rhodobacterales Rhodobacteraceae						
Site	ANOVA	F= 22	Pval < 0.001			
	MNS	CAP	PAS	PLA	PTE	TE
Station						
MNS	–					
CAP	> 0.05	–				
PAS	< 0.001	< 0.001	–			
PLA	0.003	0.005	0.01	–		
PTE	> 0.05	> 0.05	0.002	> 0.05	–	
TE	< 0.001	< 0.001	> 0.05	0.03	0.006	–
ES	< 0.001	< 0.001	0.02	< 0.001	< 0.001	< 0.001
Season	ANOVA	F= 0.617	Pval = 0.54			
Season						
Cold 2019	–					
Warm 2020	> 0.05	–				
Cold 2020	> 0.05	> 0.05	–			
Specie	ANOVA	F= 71.09	Pval < 0.001			
Season	Ha	HI	Sc			
Ha	–					
HI	< 0.001	–				
Sc	< 0.001	0.01	–			

Supplementary material 26: Inter-site and inter-seasonal variability of benthic bacteria Sphingomonadales Sphingomonadaceae in the 'ingestion' nutritional item. Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold.

Ingestion: Sphingomonadales Sphingomonadaceae						
Site	ANOVA	F= 5.474	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	< 0.001	0.002	–			
PLA	> 0.05	> 0.05	0.007	–		
PTE	> 0.05	> 0.05	< 0.001	> 0.05	–	
TE	> 0.05	> 0.05	0.01	> 0.05	> 0.05	–
ES	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05
Season	ANOVA	F= 57.62	Pval < 0.001			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	> 0.05	–				
Cold 2020	< 0.001	< 0.001	–			
Specie	ANOVA	F= 30.46	Pval < 0.001			
Season	Ha	HI	Sc			
Ha	–					
HI	< 0.001	–				
Sc	> 0.05	< 0.001	–			

Supplementary material 27: Contribution of 'Order Family' involved in the significant difference between benthic bacteria of 'available resources' and 'ingestion' items for *S. chloronotus*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated.

	Contribution %	Cumulative %	Mean 'available resources'	Mean 'ingestion' process
PAS 2019 Cold				
Pirellulales Pirellulaceae	44.03	44.03	571	8240
Rhodobacterales Rhodobacteraceae	12.22	56.25	287	2400
PAS 2020 Warm				
Pirellulales Pirellulaceae	29	29	1070	8000
Rhodobacterales Rhodobacteraceae	12.05	41.05	397	3230
Cyanobacteriales Xenococcaceae	9.536	50.59	352	2570
PAS 2020 Cold				
Sphingomonadales Sphingomonadaceae	34.16	34.16	1190	9870
Pirellulales Pirellulaceae	22.47	56.63	946	7320
TE 2019 Cold				
Pirellulales Pirellulaceae	44.05	44.05	543	8860
Rhodobacterales Rhodobacteraceae	14.66	58.72	232	2960
TE 2020 Warm				
Pirellulales Pirellulaceae	24.25	24.25	690	5610
Rhodobacterales Rhodobacteraceae	14.54	38.79	321	3370
Chloroplast Chloroplast	11.96	50.75	518	3080
TE 2020 Cold				
Sphingomonadales Sphingomonadaceae	34	34	382	8500
Pirellulales Pirellulaceae	20.84	54.84	799	6310
ES 2019 Cold				
Pirellulales Pirellulaceae	29	29	438	5450
Rhodobacterales Rhodobacteraceae	15	43.99	262	2820
Sphingomonadales Sphingomonadaceae	4.633	48.63	64	854
Cyanobacteriales Xenococcaceae	4.543	53.17	460	750
ES 2020 Warm				
Pirellulales Pirellulaceae	28.29	28.29	839	6880
Rhodobacterales Rhodobacteraceae	18.06	46.35	443	4360
Cyanobacteriales Xenococcaceae	7.267	53.62	643	1960
ES 2020 Cold				
Sphingomonadales Sphingomonadaceae	26.73	26.73	155	7110
Bacillales Bacillaceae	17.56	44.29	152	4700
Pirellulales Pirellulaceae	16.19	60.48	503	4650

Supplementary material 28: Contribution of 'Order Family' involved in the significant difference between benthic bacteria of 'available resources' and 'ingestion' item for *H. atra*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated.

	Contribution %	Cumulative %	Mean 'available resources ,	Mean 'ingestion ' process
PLA 2019 Cold				
Pirellulales Pirellulaceae	24.53	24.53	2260	6510
Sphingomonadales Sphingomonadaceae	12.44	36.97	216	2510
Cyanobacteriales Xenococcaceae	11.25	48.22	955	2540
Chloroplast Chloroplast	7.305	55.53	1440	1990
PLA 2020 Warm				
Pirellulales Pirellulaceae	15.48	15.48	1240	4610
Cyanobacteriales Xenococcaceae	15.04	30.52	599	3890
Cyanobacteriales Cyanobacteriaceae	11	41.53	300	2740
Chloroplast Chloroplast	8.714	50.24	1410	3290
PLA 2020 Cold				
Sphingomonadales Sphingomonadaceae	25.69	25.69	144	6100
Pirellulales Pirellulaceae	24.08	49.77	1040	6520
Rhodobacterales Rhodobacteraceae	6.861	56.63	488	2030

Supplementary material 29: Contribution of 'Order Family' involved in the significant difference between benthic bacteria of 'available resources' and 'ingestion' items for *H. leucospilota*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated.

	Contribution %	Cumulative %	Mean 'available resources'	Mean 'ingestion' process
MNS 2020 Warm				
Pirellulales Pirellulaceae	17.96	17.96	429	3060
Cyanobacteriales Xenococcaceae	10.20	28.16	367	1500
Flavobacteriales Cryomorpaceae	8.30	36.46	18	1270
Bacillales Bacillaceae	6.81	43.26	2	898
Chloroplast Chloroplast	6.57	49.83	377	1330
Rhodobacterales Rhodobacteraceae	3.91	53.74	167	726
MNS 2019 Cold				
Pirellulales Pirellulaceae	21.94	21.94	1230	4930
Bacillales Bacillaceae	10.46	32.39	70	1740
Cyanobacteriales Xenococcaceae	9.72	42.11	336	1690
Rhodobacterales Rhodobacteraceae	6.27	48.38	413	1450
Pseudomonadales Pseudomonadaceae	5.78	54.16	29	933
CAP 2019 Cold				
Pirellulales Pirellulaceae	18.88	18.88	1420	4700
Bacillales Bacillaceae	13.46	32.34	12	3230
Cyanobacteriales Xenococcaceae	9.85	42.19	327	1650
Rhodobacterales Rhodobacteraceae	6.14	48.34	493	1680
Spirochaetales Spirochaetaceae	5.89	54.23	17	928
CAP 2020 Warm				
Pirellulales Pirellulaceae	12.02	12.02	400	1990
Cyanobacteriales Xenococcaceae	11.73	23.75	144	1590
Chloroplast Chloroplast	8.01	31.76	514	1480
Alteromonadales Moritellaceae	6.11	37.87	21	805
Cyanobacteriales Microcystaceae	5.01	42.88	18	655
Vibrionales Vibrionaceae	4.81	47.69	4	568
Rhodobacterales Rhodobacteraceae	4.54	52.23	125	723
CAP 2020 Cold				
Bacillales Bacillaceae	23.28	23.28	13	5560
Pirellulales Pirellulaceae	13.72	37.00	717	3560
Cyanobacteriales Xenococcaceae	9.58	46.58	448	2060
Sphingomonadales Sphingomonadaceae	6.49	53.07	136	1120
PLA 2019 Cold				
Pirellulales Pirellulaceae	23.76	23.76	1810	5690
Cyanobacteriales Cyanobacteriaceae	8.23	31.99	47	1220
Chloroplast Chloroplast	7.21	39.20	1580	2050

Sphingomonadales Sphingomonadaceae	6.54	45.74	82	1090
Rhodobacterales Rhodobacteraceae	6.00	51.74	792	1630
PLA 2020 Warm				
Cyanobacteriales Xenococcaceae	19.19	19.19	479	3530
Pirellulales Pirellulaceae	9.34	28.53	394	1940
Chloroplast Chloroplast	9.16	37.69	836	2280
Rhodobacterales Rhodobacteraceae	8.73	46.43	300	1750
Cyanobacteriales Cyanobacteriaceae	5.25	51.68	292	1010
PLA 2020 Cold				
Pirellulales Pirellulaceae	14.84	14.84	1040	4110
Bacillales Bacillaceae	11.14	25.98	16	2350
Cyanobacteriales Xenococcaceae	8.14	34.13	236	2020
Rhodobacterales Rhodobacteraceae	8.14	42.27	418	2140
Chloroplast Chloroplast	6.73	49.00	1090	2300
Sphingomonadales Sphingomonadaceae	5.12	54.12	188	814
PTE 2019 Cold				
Pirellulales Pirellulaceae	18.98	18.98	1140	3800
Cyanobacteriales Xenococcaceae	15.04	34.03	444	2190
Bacillales Bacillaceae	11.81	45.84	14	1600
Chloroplast Chloroplast	7.67	53.51	1350	1980
PTE 2020 Warm				
Pirellulales Pirellulaceae	14.96	14.96	445	2620
Cyanobacteriales Xenococcaceae	14.48	29.44	480	2680
Chloroplast Chloroplast	10.95	40.39	734	2270
Rhodobacterales Rhodobacteraceae	8.16	48.55	238	1450
Fusobacteriales Fusobacteriaceae	4.11	52.67	2	630
PTE 2020 Cold				
Bacillales Bacillaceae	21.42	21.42	131	4930
Pirellulales Pirellulaceae	16.48	37.90	621	3810
Cyanobacteriales Xenococcaceae	12.45	50.36	273	2450
TE 2019 Cold				
Pirellulales Pirellulaceae	26.98	26.98	1030	5320
Rhodobacterales Rhodobacteraceae	7.84	34.81	229	1530
Chloroplast Chloroplast	6.51	41.32	581	1590
Bacteroidales	6.41	47.73	1	1050
Cyanobacteriales Xenococcaceae	5.66	53.39	39	836
TE 2020 Warm				
Pirellulales Pirellulaceae	15.53	15.53	654	2730
Chloroplast Chloroplast	12.34	27.87	600	2250
Cyanobacteriales Xenococcaceae	9.05	36.92	90	1370
Rhodobacterales Rhodobacteraceae	9.04	45.96	245	1460
Bacillales Bacillaceae	7.44	53.40	21	947

TE 2020 Cold

Pirellulales Pirellulaceae	26.12	26.12	1100	5260
Rhodobacterales Rhodobacteraceae	12.03	38.15	364	2280
Bacillales Bacillaceae	10.31	48.46	9	2070
Cyanobacteriales Xenococcaceae	6.50	54.96	243	1190

Supplementary material 30: Benthic bacteria involved in the ‘assimilation’ nutritional item inter-site variability. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold.

HI	ANOSIM $R= 0.085$ $Pval < 0.001$				
Station	MNS	CAP	PLA	PTE	TE
MNS	–				
CAP	0.01	–			
PLA	0.01	0.01	–		
PTE	0.01	0.01	0.02	–	
TE	0.01	0.01	0.01	0.01	–
Sc	ANOSIM $R= 0.044$ $Pval < 0.001$				
Station	PAS	TE	ES		
PAS	–				
TE	0.426	–			
ES	0.003	0.003	–		

Supplementary material 31: Intra-site seasonal variability of benthic bacteria involved in the ‘assimilation’ nutrition items. Results of ANOSIM and pairwise ANOSIM for each site. Significant Pvalue are indicated in bold.

MNS HI	ANOSIM	<i>R</i> = 0.096	<i>P</i> val < 0.001
CAP HI	ANOSIM	<i>R</i> = 0.239	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.009	–	
2020 Cold	0.015	0.003	–
PAS Sc	ANOSIM	<i>R</i> = 0.277	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.084	–	
2020 Cold	0.003	0.003	–
PLA Ha	ANOSIM	<i>R</i> = 0.281	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.003	0.003	–
PLA HI	ANOSIM	<i>R</i> = 0.207	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.009	0.003	–
PTE HI	ANOSIM	<i>R</i> = 0.137	<i>P</i> val = 0.008
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.393	–	
2020 Cold	0.162	0.048	–
TE HI	ANOSIM	<i>R</i> = 0.219	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.003	0.012	–
TE Sc	ANOSIM	<i>R</i> = 0.239	<i>P</i> val < 0.001
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.003	–	
2020 Cold	0.003	0.003	–
ES Sc	ANOSIM	<i>R</i> = 0.125	<i>P</i> val = 0.002
Season	2019 Cold	2020 Warm	2020 Cold
2019 Cold	–		
2020 Warm	0.012	–	
2020 Cold	0.006	0.147	–

Supplementary material 32: Inter-site and inter-seasonal variability of benthic bacteria *Pirellulales Pirellulaceae* in the ‘assimilation’ nutritional item. Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold.

Assimilation: Pirellulales Pirellulaceae						
Site	ANOVA	F= 20.56	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	< 0.001	< 0.001	–			
PLA	> 0.05	0.01	< 0.001	–		
PTE	> 0.05	> 0.05	< 0.001		–	
TE	> 0.05	0.05	< 0.001			–
ES	< 0.001	< 0.001	> 0.05	< 0.001	< 0.001	< 0.001
Season	ANOVA	F= 4.012	Pval = 0.018			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	> 0.05	–				
Cold 2020	0.01	> 0.05	–			
Specie	ANOVA	F= 131.3	Pval < 0.001			
Season	Ha	HI	Sc			
Ha	–					
HI	< 0.001	–				
Sc	< 0.001	< 0.001	–			

Supplementary material 33: Inter-site and inter-seasonal variability of benthic bacteria Rhodobacterales Rhodobacteraceae in the ‘assimilation’ nutritional item. Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold.

Assimilation: Rhodobacterales Rhodobacteraceae						
Site	ANOVA	F= 22.12	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	< 0.001	< 0.001	–			
PLA	> 0.05	> 0.05	< 0.001	–		
PTE	> 0.05	> 0.05	0.03		–	
TE	0.002	< 0.001	> 0.05	0.005	> 0.05	–
ES	< 0.001	< 0.001	0.004	< 0.001	< 0.001	< 0.001
Season	ANOVA	F= 6.625	Pval = 0.001			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	> 0.05	–				
Cold 2020	< 0.001	> 0.05	–			
Specie	ANOVA	F= 87.28	Pval < 0.001			
Season	Ha	HI	Sc			
Ha	–					
HI	> 0.05	–				
Sc	< 0.001	< 0.001	–			

Supplementary material 34: Inter-site and inter-seasonal variability of benthic bacteria Sphingomonadales Sphingomonadaceae in the ‘assimilation’ nutritional item. Results of ANOVA and Tukey HSD. Significant *P*value are indicated in bold.

Assimilation: Sphingomonadales Sphingomonadaceae						
Site	ANOVA	F= 9.745	Pval < 0.001			
Station	MNS	CAP	PAS	PLA	PTE	TE
MNS	–					
CAP	> 0.05	–				
PAS	< 0.001	< 0.001	–			
PLA	> 0.05	> 0.05	< 0.001	–		
PTE	> 0.05	> 0.05	< 0.001	> 0.05	–	
TE	> 0.05	> 0.05	< 0.001	> 0.05	> 0.05	–
ES	> 0.05	> 0.05	< 0.001	> 0.05	> 0.05	> 0.05
Season	ANOVA	F= 30.82	Pval < 0.001			
Season	Cold 2019	Warm 2020	Cold 2020			
Cold 2019	–					
Warm 2020	> 0.05	–				
Cold 2020	< 0.001	< 0.001	–			
Specie	ANOVA	F= 24.3	Pval < 0.001			
Season	Ha	HI	Sc			
Ha	–					
HI	0.009	–				
Sc	> 0.05	< 0.001	–			

Supplementary material 35: Contribution of 'Order Family' involved in the significant difference between benthic bacteria of 'ingestion' and 'assimilation' nutritional items for *S. chloronotus*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated

	Contribution %	Cumulative %	Mean 'ingestion' process	Mean 'assimilation' process
PAS 2019 Cold				
Pirellulales Pirellulaceae	37.05	37.05	8240	2720
Rhodobacterales Rhodobacteraceae	10.70	47.75	2400	811
Cyanobacteriales Xenococcaceae	6.19	53.94	1140	267
PAS 2020 Warm				
Pirellulales Pirellulaceae	23.41	23.41	8000	2900
Cyanobacteriales Xenococcaceae	11.06	34.47	2570	0
Rhodobacterales Rhodobacteraceae	10.12	44.59	3230	1010
Bacillales Bacillaceae	9.28	53.87	2340	0
PAS 2020 Cold				
Sphingomonadales Sphingomonadaceae	30.86	30.86	9870	5370
Pirellulales Pirellulaceae	20.52	51.38	7320	2090
TE 2019 Cold				
Pirellulales Pirellulaceae	33.18	33.18	8860	3760
Rhodobacterales Rhodobacteraceae	14.14	47.32	2960	820
Chloroplast Chloroplast	9.24	56.56	1560	167
TE 2020 Warm				
Pirellulales Pirellulaceae	19.61	19.61	5610	2270
Chloroplast Chloroplast	13.03	32.64	3080	707
Rhodobacterales Rhodobacteraceae	12.45	45.09	3370	1070
Bacillales Bacillaceae	8.31	53.40	1350	0
TE 2020 Cold				
Sphingomonadales Sphingomonadaceae	31.36	31.36	8500	3710
Pirellulales Pirellulaceae	20.19	51.55	6310	1890
ES 2019 Cold				
Pirellulales Pirellulaceae	20.99	20.99	5450	2610
Rhodobacterales Rhodobacteraceae	13.09	34.08	2820	935
Chloroplast Chloroplast	6.47	40.55	1120	160
Sphingomonadales Sphingomonadaceae	4.96	45.51	854	627
Bacillales Bacillaceae	4.52	50.04	605	0
ES 2020 Warm				
Pirellulales Pirellulaceae	22.45	22.45	6880	3220
Rhodobacterales Rhodobacteraceae	15.56	38.01	4360	1430

Cyanobacteriales Xenococcaceae	9.09	47.10	1960	0
Flavobacteriales Flavobacteriaceae	6.64	53.74	1490	219
ES 2020 Cold				
Sphingomonadales	28.37	28.37	7110	0
Sphingomonadaceae				
Bacillales Bacillaceae	17.96	46.33	4700	0
Pirellulales Pirellulaceae	13.91	60.24	4650	1770

Supplementary material 36: Contribution of 'Order Family' involved in the significant difference between benthic bacteria of 'ingestion' and 'assimilation' nutritional items for *H. atra*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated.

	Contribution %	Cumulative %	Mean 'ingestion' process	Mean 'assimilation' process
PLA 2019 Cold				
Pirellulales Pirellulaceae	24.82	24.82	6510	1670
Sphingomonadales Sphingomonadaceae	11.41	36.23	2510	0
Cyanobacteriales Xenococcaceae	11.16	47.39	2540	584
Chloroplast Chloroplast	10.8	58.19	1990	0
PLA 2020 Warm				
Cyanobacteriales Xenococcaceae	16.2	16.2	3890	0
Pirellulales Pirellulaceae	13.81	30.02	4610	1560
Cyanobacteriales Cyanobacteriaceae	10.84	40.86	2740	0
Chloroplast Chloroplast	10.73	51.59	3290	695
PLA 2020 Cold				
Sphingomonadales Sphingomonadaceae	21.13	21.13	6100	2070
Pirellulales Pirellulaceae	18.76	39.9	6520	2170
Chloroplast Chloroplast	11.42	51.31	2700	0

Supplementary material 37: Contribution of 'Order Family' involved in the significant difference between benthic bacteria of 'ingestion' and 'assimilation' nutritional items for *H. leucospilota*. Results of SIMPER analyses. Results present the major contributions up to 50% cumulated

	Contribution %	Cumulative %	Mean 'ingestion' process	Mean 'assimilation' process
MNS 2020 Warm				
Pirellulales Pirellulaceae	17.32	17.32	3060	603
Cyanobacteriales Xenococcaceae	9.568	26.89	1500	241
Flavobacteriales Cryomorpaceae	8.009	34.89	1270	17
Chloroplast Chloroplast	7.042	41.94	1330	264
Bacillales Bacillaceae	6.573	48.51	898	0
Rhodobacterales Rhodobacteraceae	3.615	52.12	726	205
MNS 2020 Cold				
Pirellulales Pirellulaceae	22.15	22.15	4930	666
Bacillales Bacillaceae	11.61	33.75	1740	867
Cyanobacteriales Xenococcaceae	9.908	43.66	1690	0
Rhodobacterales Rhodobacteraceae	6.058	49.72	1450	287
Pseudomonadales Pseudomonadaceae	5.267	54.99	933	0
CAP 2019 Cold				
Pirellulales Pirellulaceae	20.91	20.91	4700	767
Bacillales Bacillaceae	12.61	33.51	3230	0
Cyanobacteriales Xenococcaceae	9.653	43.17	1650	247
Rhodobacterales Rhodobacteraceae	7.053	50.22	1680	191
CAP 2020 Warm				
Pirellulales Pirellulaceae	12.6	12.6	1990	178
Cyanobacteriales Xenococcaceae	12.54	25.13	1590	0
Chloroplast Chloroplast	9.829	34.96	1480	287
Alteromonadales Moritellaceae	6.05	41.01	805	0
Cyanobacteriales Microcystaceae	4.978	45.99	655	0
Vibrionales Vibrionaceae	4.696	50.69	568	0
CAP 2020 Cold				
Bacillales Bacillaceae	21.12	21.12	5560	0
Pirellulales Pirellulaceae	15.96	37.09	3560	0
Cyanobacteriales Xenococcaceae	9.126	46.21	2060	297
Sphingomonadales Sphingomonadaceae	6.317	52.53	1120	0
PLA 2019 Cold				
Pirellulales Pirellulaceae	25.5	25.5	5690	729
Chloroplast Chloroplast	10.17	35.67	2050	236
Cyanobacteriales Cyanobacteriaceae	7.793	43.46	1220	0

Rhodobacterales Rhodobacteraceae	7.743	51.21	1630	237
PLA 2020 Warm				
Cyanobacteriales Xenococcaceae	20.43	20.43	3530	0
Chloroplast Chloroplast	9.451	29.88	2280	644
Pirellulales Pirellulaceae	9.348	39.23	1940	456
Rhodobacterales Rhodobacteraceae	7.503	46.73	1750	365
Cyanobacteriales Cyanobacteriaceae	5.592	52.32	1010	0
PLA 2020 Cold				
Pirellulales Pirellulaceae	15.65	15.65	4110	639
Bacillales Bacillaceae	10.19	25.84	2350	0
Cyanobacteriales Xenococcaceae	8.478	34.32	2020	0
Chloroplast Chloroplast	8.205	42.53	2300	475
Rhodobacterales Rhodobacteraceae	7.609	50.14	2140	383
PTE 2019 Cold				
Pirellulales Pirellulaceae	18.99	18.99	3800	626
Cyanobacteriales Xenococcaceae	16.32	35.32	2190	0
Chloroplast Chloroplast	10.69	46	1980	339
Bacillales Bacillaceae	10.4	56.41	1600	0
PTE 2020 Warm				
Cyanobacteriales Xenococcaceae	15.9	15.9	2680	0
Pirellulales Pirellulaceae	14.67	30.58	2620	1060
Chloroplast Chloroplast	11.74	42.32	2270	513
Rhodobacterales Rhodobacteraceae	7.765	50.08	1450	536
PTE 2020 Cold				
Bacillales Bacillaceae	23.4	23.4	4930	2360
Pirellulales Pirellulaceae	15.27	38.67	3810	659
Cyanobacteriales Xenococcaceae	12.88	51.55	2450	0
TE 2019 Cold				
Pirellulales Pirellulaceae	29.82	29.82	5320	0
Chloroplast Chloroplast	8.577	38.4	1590	156
Rhodobacterales Rhodobacteraceae	7.599	46	1530	149
Cyanobacteriales Xenococcaceae	5.469	51.46	836	0
TE 2020 Warm				
Pirellulales Pirellulaceae	16.06	16.06	2730	294
Chloroplast Chloroplast	12.93	28.99	2250	381
Cyanobacteriales Xenococcaceae	8.761	37.75	1370	0
Rhodobacterales Rhodobacteraceae	8.07	45.82	1460	288
Bacillales Bacillaceae	6.819	52.64	947	0
TE 2020 Cold				
Pirellulales Pirellulaceae	29.33	29.33	5260	0
Rhodobacterales Rhodobacteraceae	11.37	40.71	2280	251
Bacillales Bacillaceae	9.052	49.76	2070	0
Pseudomonadales Moraxellaceae	6.106	55.86	928	590

Chapitre 5 –

Stress physiologique chez les populations d'holothuries en réponse à la pollution anthropique



Individus de Holothuria leucospilota au sein de l'herbier monospécifique de Syringodium isoetifolium dans le récif de l'Hermitage

12.02.2020

Résumé

La pollution des récifs coralliens est croissante depuis plusieurs décennies et peut prendre plusieurs types, comme la pollution organique ou par les plastiques. La mesure de telle pollution est difficile *in situ* et le développement de biomarqueur est en plein essor. Il s'agit alors de quantifier une réponse physiologique chez des organismes qui renseignent sur l'état de pollution du milieu. Pour être robuste, les approches multibiomarqueurs, multispécifiques et multi-sites sont mises en avant pour décrire de manière exhaustive la pollution. Les holothuries dépositives sont rarement étudiées et considérées comme espèce indicatrice, bien qu'elles possèdent des qualités adaptées. En effet, elles sont largement distribuées dans le monde, ingèrent de grande quantité de sédiment et ont une faible mobilité.

À la Réunion, plusieurs études renseignent sur l'état dégradé du récif qui est sujet à des résurgences d'eaux douces riches en nutriments et en pesticides. L'agriculture et les activités humaines sont mises en cause dans cette pollution.

Ce chapitre vise à développer, dans une approche 'multi', deux nouveaux biomarqueurs liés aux dommages oxydatifs des protéines et de les doser dans les tissus tégumentaires des trois espèces d'holothurie, présentes dans plusieurs endroits du récif. En parallèle, il s'agit d'établir le lien avec le dosage de l'acétylcholinestérase, biomarqueur largement utilisé en routine chez de nombreuses espèces marines pour faire évaluer la réponse neurale à la toxicité engendrée par une pollution organique par les pesticides. Ainsi, le stress physiologique des trois espèces d'holothuries sera quantifié et permettra de renseigner si les facteurs anthropiques, tel que la pollution par les pesticides, ont une influence sur leur distribution en aggrégation.

Les résultats de ce chapitre sont en préparation pour être soumis dans *Marine Pollution Bulletin*.

Sensitive and resistant to pollution? Innovative combination of multi-biomarkers reveals sea cucumbers as potent bioindicators of pollution

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Abstract

Research of biomarkers related to pollutant levels represents an important methodological challenge for the assessment and monitoring of environmental stress in populations of marine taxa. Here, an innovative multi-biomarkers combination was tested: neural response to toxicity, and oxidative protein damage from exposure were evaluated by measuring acetylcholinesterase (AChE) and advanced oxidative end products (AOPP) levels with chymotrypsin-like activity of the proteasome (LLVY) in wild sea cucumber populations ($N=135$). Sampling design was conducted through three consecutive seasons over seven sites at Reunion Island. Correlations were used to investigate potential link between AChE, AOPP

and LLVY. Then, multiple linear regressions were applied to understand the link between biomarkers and individuals and ecological variables. The trends for the three biomarkers were congruent, with the proteasomal activity and acetylcholinesterase biomarkers highly correlated. Moreover, all three biomarkers exhibited same responses in terms of temporal, spatial, species-related, individual and ecological variables. Our results highlight that sea cucumbers are both sensitive and resistant to organic pollution. Sensitivity is at individual level when resistance is at population level. First, long-term monitoring of AChE revealed that they can present a wide range of AChE values and have a high tolerance threshold. Then, spatial variability observed among sites are in favour of a significant sensitivity of sea cucumbers to organic pollution. Finally, sea cucumber populations appeared healthy in Reunion Island, as attested by the low and stable values of AChE, AOPP and LLVY measured, and by the high densities of sea cucumbers and population stability. Further research on environment stress impact on wild sea cucumber remains highly warranted. In conclusion, our results clearly highlight that (1) oxidative protein damage can be used as innovative biomarkers to assessing organic pollution in marine taxa and (2) sea cucumbers are a good indicator species covering a wide spectrum of organic pollution.

Keywords

Sea cucumber • Multi-biomarkers approach • Oxidative protein damage • Acetylcholinesterase • Pollution • Indicator species

Introduction

Coral reefs represent less than 0.1% of the world ocean floor, with an estimated area about 527,072 km² (Mora *et al.*, 2006). They ensure ecosystem health through the protection of coast or food stocks, as well as the preservation of social and cultural services (Moberg & Folke, 1999; Woodhead *et al.*, 2019), which have been estimated to \$35.8 billions a year (Spalding *et al.*, 2017). However, coral reefs are threatened worldwide by many factors such as overfishing, climate change or pollution (Riegl *et al.*, 2009; van Dam *et al.*, 2011; Frieler *et al.*, 2013). At least 25% of coral reefs have already disappeared (Carpenter *et al.*, 2008) and their capacity to provide ecosystem services has declined by half since the 1950s (Eddy *et al.*,

2021). Pollution of coral reefs is mainly due to the groundwater, sewage discharges and rainwater runoffs that increase nutrient, sediment and toxic substance loads in reef waters leading to eutrophication, metabolic changes and decrease rates of growth and reproduction of marine organisms .

Agriculture constitutes a key sector for the economy of Reunion Island, with approximately 38,650 ha of filed, including 21,350 ha allocated to sugar cane production (DAAF Réunion, 2021). A total of 193 types of pesticides have been imported in Reunion Island between 2009 and 2011, with 11 herbicides specialized in the agriculture of sugar cane, which represented 120 T.year⁻¹ of active substances (Martin *et al.*, 2013). Almost all these active substances are classified 'N', according to the European Commission (CE), meaning that they are "very toxic to aquatic organisms, which may cause long-term adverse effects in the aquatic environment". In consequence, the chemical weed control of agriculture was classified as the first local phytosanitary problem in Reunion Island (Martin *et al.*, 2013). Groundwater discharges represent the main source of freshwater affecting the coral reefs of L'Hermitage/La Saline and Etang-Salé, in the west coast of Reunion Island (Join, 1991). In 2020, triazine herbicides, such as atrazine or terbuthylazin, organochlorine pesticide, such as metolachlor, and heavy metals, such as lead and copper, were recorded in reef waters of Reunion Island (Office de l'eau Réunion, 2022). Moreover, the use of fertilizer has increased the quantity of phosphorous and nitrogen compounds (Savci, 2012), leading to the eutrophication of the reefs (Cuet *et al.*, 1988; Cuvillier, 2016). In conclusion, Reunion Island reefs appear to be eutrophic and polluted by pesticides.

Coral reefs of Reunion Island host almost 40 species of sea cucumbers (Conand *et al.*, 2018). Three species have a patchy distribution with very high densities at some locations, for such large invertebrates: *Holothuria leucospilota* (up to 2 ind.m² Cuvillier, 2016), *Holothuria atra* (up to 6.6 ind.m², Conand, 2004, 1995) and *Stichopus chloronotus* (up to 3.8 ind.m², Conand *et al.*, 1998). Sea cucumbers have ecological and physiological characteristics to make them interesting marine indicator taxa (Warnau *et al.*, 2006) for pollution. They have a global distribution, from polar to tropical latitudes and from shallow to abyssal depths. Most of sea cucumbers are sessile organisms, with very low mobility (Taddei, 2006). They ingest big

amounts of sediment (Yamanouchi, 1939; Mangion *et al.*, 2004) that are processed through their guts and then excreted. For instance, the three high-density species in Reunion Island, process very high amounts of sediment, up to 90 ± 46 and 99 ± 30 g.dw.ind⁻¹.d⁻¹ for *H. atra* and *H. leucospilota* (Mangion *et al.*, 2004) and up to 59 g.dw.ind⁻¹.d⁻¹ for *S. chloronotus* (Uthicke, 1999). Sea cucumbers also pump large amounts of water through the anus for breathing and even feeding on suspended matter (Jaeckle and Strathmann, 2013; see reviewed by Pierrat *et al.*, 2022). Finally, they are among the most important benthic taxa in deep-sea (Kuhnz *et al.* 2014), corals reefs (Wolfe & Davey, 2020) and shallow marine habitats (Conde *et al.*, 1991). Only few studies have considered sea cucumbers as bioindicators (Warnau *et al.*, 2006; Kolasinski *et al.*, 2010), and more studies are needed to test a large range of biomarkers to confirm their relevance as indicators. Regarding to the pollution recorded in Reunion Island reefs, and the presence of wild high-density sea cucumber populations, Reunion Island appears to be an ideal locality to conduct multi-biomarkers studies on sea cucumbers for pollution monitoring.

A multi-biomarkers approach is one of the main methods currently used to evaluate reef level of pollution (Linde-Arias *et al.*, 2008; Gusso-Choueri *et al.*, 2015). Biomarkers quantify the level of exposure to organic pollution by assessing the stress response of marine taxa, at several levels (molecular, cellular, physiological or genetic). Biomarkers can be separated in two groups: biomarkers of exposure which are involved in the activation of regulatory mechanism and biomarkers of toxic effects which highlight an overrun of the regulatory capacities of the organism, possibly with consequences on the viability (cell, tissue, organ or individual) (Vasseur & Cossu-Leguille, 2003). A multi-biomarkers approach applied on several individuals from various body parts of different species is necessary to characterize the level of pollution from individual to populational and ecosystem scales.

Some biomarkers are widely used to assess marine taxa physiology, such as acetylcholinesterase (AChE), that reflects neurotoxic impacts mainly due to organism exposition to organophosphate and carbamate pesticide pollution (Weiss, 1958; Fu *et al.*, 2018; Umar & Aisami, 2020). AChE is therefore considered as a biomarker of exposure (Vasseur & Cossu-Leguille, 2003). AChE has been assessed in many wild populations of marine

taxa, such as bivalves (Matozzo, Tomei & Marin, 2005), fish (de la Torre, Ferrari & Salibián, 2002), polychaetes (Pérez, Blasco & Solé, 2004), copepods (Forget, Beliaeff & Bocquené, 2003) or echinoderms (Den Besten *et al.*, 2001; Cunha, García & Guilhermino, 2005). To our knowledge, only one study has evaluated AChE in wild sea cucumber populations (Kolasinski *et al.*, 2010).

Biomarkers of oxidative stress, such as antioxidant enzyme activities (e.g. catalase, superoxide dismutase, glutathione) or lipid peroxidation, are generally used to monitor the level of a global environmental pollution, which could be linked, for instance, to the presence of heavy metals (Farombi, Adelowo & Ajimoko, 2007) or microplastics (Lombardo *et al.*, 2022). They are considered as biomarkers of toxic effects (Vasseur & Cossu-Leguille, 2003) and have been assessed in numerous marine taxa, as bivalves (Box *et al.*, 2007; Jena, Verlecar & Chainy, 2009), fish (Yildirim, Benzer & Danabas, 2011; Valon *et al.*, 2013), crustaceans (Frías-Espéricueta *et al.*, 2022) or sea cucumbers (Telahigue *et al.*, 2019; Lombardo *et al.*, 2022). However, biomarkers involving oxidation protein damage are still sparsely used.

Enhanced oxidative stress may lead to the occurrence of protein oxidative damage. Because of their involvement in multiple biological functions (structure, receptors, transporters, enzymes, ...), oxidative damage to proteins can have detrimental effects *in vivo* (Stadtman & Levine, 2006). Relevant biomarkers have been developed to monitor oxidative damage to proteins *in vivo*. Advanced oxidation end products (AOPP) represent a useful biomarker, whose levels have been well correlated with ageing and tissue degeneration (Cakatay *et al.*, 2003, 2003; Altunoglu *et al.*, 2015). Elevated levels of AOPP have also recently been linked to exposure of pregnant women to fine particles (Yang *et al.*, 2023).

The proteasome constitutes a proteolytic complex involved in the degradation of oxidized proteins in cells (Vanhooren *et al.*, 2015). The chymotrypsin-like activity of the proteasome (LLVY) represents one of three particularly active in the complex. As the proteasome is responsible for oxidized protein removal, its role has received considerable attention, and there is evidence that its activities are altered in oxidative stress and aging conditions (Vanhooren *et al.*, 2015), therefore the LLVY start to be used as biomarker of oxidative protein damage.

As far as we know, the biomarkers AOPP and LLVY have never been used to assess environmental stress in wild populations of marine taxa. The general objective of this study is to assess their efficiency as indicators of stress in wild sea cucumber populations under environmental disturbances and then strengthen the multi-biomarker approach in those populations. A multi-biomarker approach with AChE, AOPP and LLVY biomarkers appear to be an optimal combination with biomarkers covering both exposure (AChE) and toxicity effect (protein damage) (Vasseur & Cossu-Leguille, 2003). As AChE has proved to be informative about sea cucumber populations stress level in Reunion Island (Kolasinski *et al.*, 2010), it can be used as a reference biomarker in this location. Our study aims then to establish a correlation between oxidative protein damage and neural response to toxicity given by AChE quantification in sea cucumbers. Finally, another specific objective is to understand the link between these biomarkers and individual and ecological variables. The results should bring a new insight on the use of sea cucumber as pollution indicators.

Materials and Methods

Study area

Reunion Island (France), located in the South Western of the Indian Ocean (21°06'S, 55°31'E), is part of the Mascareignes archipelago with Mauritius and Rodrigues. It is a young volcanic Island, about three million years old (Duncan *et al.*, 1989), with a dense hydrographic network (Join, 1991). Reunion Island has a humid tropical climate characterized by the alternance of two seasons: the austral summer (October to March) is warm and humid and the austral winter (April to September) is cold and dry (Conand *et al.*, 2007). Fringing reefs of Reunion Island cover an area of 12 km² and are divided into four reefs distributed to the West and South coasts (Montaggioni & Faure, 1980).

Sampling design

Sampling was carried out in the West coast, in the back reefs of L'Hermitage/La Saline and Étang-Salé (**Figure 30**). Individuals of three sea cucumber species were randomly sampled by hand collecting. Individuals of *H. leucospilota* were collected in five sites throughout the

fringing reef of L'Hermitage/La Saline. A first group of three sites, named MNS (Maîtres Nageurs Sauveteurs), PLA (Planch'Alizé) and TE (Trou d'Eau) where high densities are recorded ($> 1 \text{ ind.m}^2$, Conand and Mangion, 2002; Cuvillier, 2016) and two sites, named CAP (Cap-Méchant) and PTE (Petit Trou d'Eau), with low densities ($< 1 \text{ ind.m}^2$, personal observations). *Holothuria atra* were only present and sampled at PLA, where they have high density (Conand & Mangion, 2002). Finally, individuals of *S. chloronotus* were collected at PAS (Passe de l'Hermitage) and TE in the fringing reef of L'Hermitage/La Saline and at ES (Étang-Salé) in the fringing reef of Étang-Salé, where they have high densities. Four of these sites have been characterized as eutrophic, PAS, PLA, TE and ES, have been receiving nutrient through enriched freshwater (Cuet et al., 1988; Cuvillier, 2016, P. Cuet personal communication). Sampling was carried out on three consecutive seasons, the austral winter 2019 and 2020 (September), the austral summer 2020 (February), to assess any seasonal or interannual effects. Five individuals were sampled at each site and each season ($N= 135$, DEAL authorization N°2020-37), regardless of the sex of individuals. In the field, they were kept in dry ice and stored later at -80°C in the laboratory before analyses.

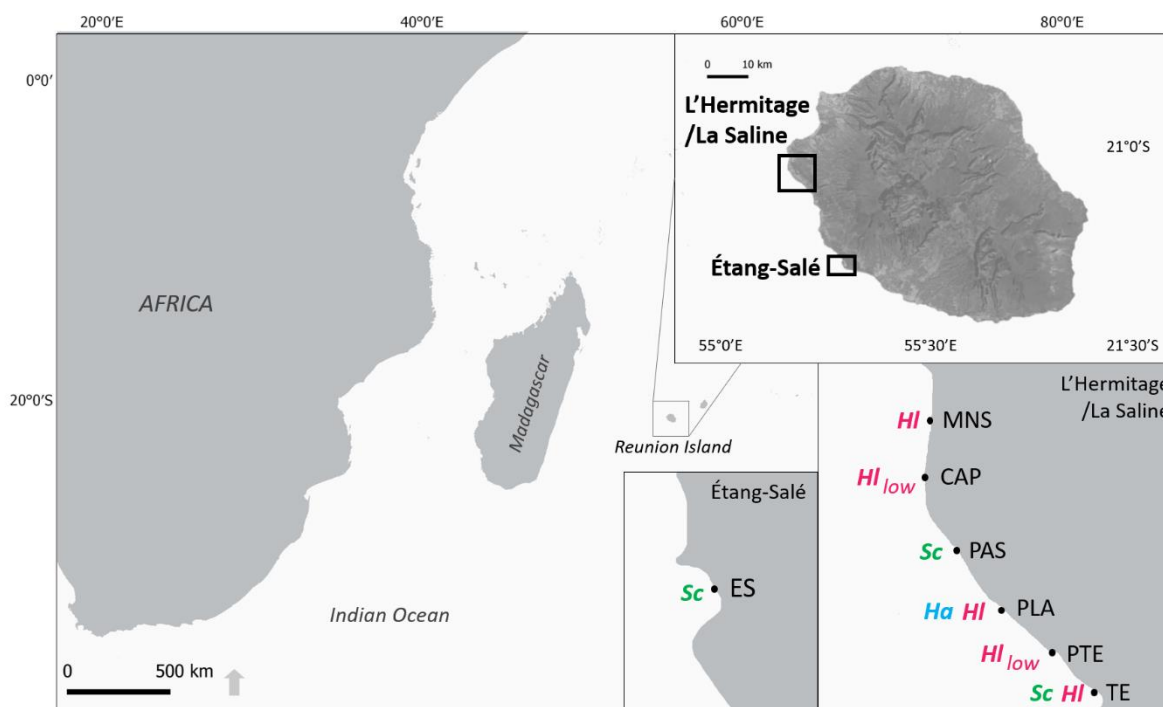


Figure 30: Location of Reunion Island in the western Indian Ocean. Location of L'Hermitage/La saline and Étang-Salé reefs in the west coast of Reunion Island, and the location of the sampling sites at each reef.

HI : *Holothuria leucospilota* ; *Ha* : *Holothuria atra* ; *Sc* : *Stichopus chloronotus*

Biomarker assays

Individuals were dissected on ice under sterile conditions. Two different body parts were taken from each individual: (1) body wall for advanced oxidation protein products (AOPP) and chymotrypsin-like activity of proteasome (LLVY) quantification, (2) longitudinal muscles for acetylcholinesterase (AChE) analyses.

(i) Oxidative protein damage

About 120 mg body wall tissues previously collected and stored at -80°C were homogenized with a TissueLyser II (Qiagen) in 1 mL Tris-HCl 25 mM, pH 7.5. After centrifugation (5000 g/min, for 10 min at 4°C), the supernatant was used for AOPP and LLVY analyses together with total protein quantification by using bicinchoninic acid assay (Smith *et al.*, 1985). Advanced oxidation end-products (AOPP) level in homogenate samples was estimated by spectrophotometry according to Capeillere-Blandin *et al.* (2004) protocol. Briefly, 200 μL of tissue homogenates diluted 1:10 in phosphate buffer (Tris-HCl 25 mM, pH

7.5), 200 μ L of chloramine T (0–100 μ mol/L, Sigma) for calibration and 200 μ L of phosphate buffer (Tris–HCl 25 mM, pH 7.5) as blank were applied on a microtiter plate. Ten microliters of 1.16 M potassium iodide and 20 μ L of acetic acid were added and absorbance was measured at 340 nm by using a microplate spectrofluorometer reader (Fluostar, BMG France). AOPP concentration was expressed in chloramine units per mg of protein.

(ii) Chymotrypsin-like activity of proteasome

Chymotrypsin-like activity of proteasome was assayed using Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin fluorogenic peptide at 25 μ M (Sigma) and as described in Le Pecheur et al. (2005). Briefly, approximately 50 μ g protein of tissue homogenate in 25 mM Tris-HCl (pH 7.5) were incubated with LLVY substrate at 37°C for 30 min. Aminomethylcoumarin product fluorescence was measured every minute at 350/440 nm excitation/emission wavelengths by using a microplate spectrofluorometer reader (Fluostar, BMG France). Peptidase activity was measured in the absence or the presence (20 μ M) of the proteasome inhibitor MG132 (N-Cbz-Leu-Leu-leucinal) and the difference between the two values was attributed to proteasome activity. LLVY proteasome activity was expressed in fluorescence arbitrary unit per mg of protein.

(iii) Acetylcholinesterase enzymatic activity

Approximately 200 mg of muscles was homogenized on ice, in ice-cold 0.02 M pH 7 phosphate buffer+0.1% Triton X100 ($\frac{1}{4}$ and $\frac{1}{3}$ volume/weight) using a Precellys Homogenizer (Bertin Technology). The homogenates were then centrifuged at 10,000 \times g for 5 min at 4°C. Measurements of AChE activity were performed using the colorimetric method of Ellman et al. (1961), modified by Bocquené and Galgani (1998) at 412 nm, with acetylthiocholine iodide (AcSCh) as substrate and dithio-bis-nitrobenzoate (DTNB) as reagent at a controlled temperature of 20°C. The extinction coefficients used to determine AChE specific activities was 12,600 $M^{-1} cm^{-1}$ at 412 nm. AChE activity is expressed as $nmol min^{-1} mg^{-1}$ protein. Proteins used for standardization were determined using Bradford (1976) method modified for use with a micro-plate reader (Bocquené & Galgani, 1998). All assays were performed in quadruplicate.

Population and environmental data

Some population variables were recorded during sampling. Each individual collected was measured (length and width). Meanwhile, sea cucumber density was assessed at each site and each season with four belt transects (2 × 50 m). Sediments were cored with triplicates over 1 m² to investigate total organic matter load by calcination (4h at 450 °C), fines particles (sieved through 0.063 mm) and chlorophyll a (90% acetone extraction). Rainfall accumulation data over three months were downloaded from Météo France website (<https://meteofrance.re/fr/climat/bulletins-annuels>), for each sampling (winter 2019, summer 2020 and winter 2020) and each reef (Hermitage-La Saline and Étang-Salé).

Statistical analysis

Individual and ecological parameters were tested for spatio-temporal variations with ANOVA. Then, correlations were investigated with linear regressions between the three biomarkers. According to their distribution, AOPP and LLVY were log₁₀ transformed. Finally, multiple linear regressions were used to understand which individuals and ecological variables influenced the most each biomarker. The general outcome variables (AChE, AOPP and LLVY) are expressed by the following equation:

$$\begin{aligned} \text{Biomarker} = & \text{density} + \text{length} + \text{season} + \text{species} + \text{site} + \\ & \text{organic matter} + \text{chlorophyll a} + \text{fine particles} + \text{rainfall accumulation} + \\ & \text{length} \times \text{species} + \text{rainfall accumulation} \times \text{site} + \text{density} \times \text{species} + \text{season} \times \\ & \text{species} + \text{organic matter} \times \text{site} + \text{organic matter} \times \text{season} + \text{chlorophyll a} \times \text{site} + \\ & \text{chlorophyll a} \times \text{season} + \text{chlorophyll a} \times \text{rainfall accumulation} + \text{fine particles} \times \\ & \text{site} + \text{fine particles} \times \text{season}. \end{aligned}$$

To find out the best model representing the data for each biomarker, we used the function *stepAIC* in the R package MASS (Venables & Ripley, 2002). Each biomarker final equation is shown in **Supplementary material 38**. Then, ANOVA were applied on each final model to find out significant factors. Afterwards, t-test or correlation test were used depending on the variables. Noteworthy, post hoc tests occasionally showed no significant differences even if

ANOVA was significant. We considered results of post hoc tests. All analyses were realized with R (R Core Team, 2019).

Results

Spatio-temporal variability for individual metrics and ecological variables

Regarding individual patterns, density and length of *H. leucospilota* were stable through time (**Table 13**), but spatial differences occurred. *Holothuria leucospilota* reached high density ($2.7 \pm 0.3 \text{ ind.m}^2$) at MNS where the individuals were the smallest ($21.6 \pm 1.2 \text{ cm}$), whereas the lowest densities were observed at CAP and PTE (less than 0.05 ind.m^2) where individuals were the longest ($31.0 \pm 2.2 \text{ cm}$ for PTE, **Supplementary material 39****Supplementary material 40**). There was a significant correlation between the length and density of individuals ($r(132) = -0.299$; $p\text{-value} < 0.001$). Temporal differences were recorded for *S. chloronotus* density (**Table 13**). During the winter 2020, the density of *S. chloronotus* was the lowest ($0.6 \pm 0.1 \text{ ind.m}^2$). Spatial differences occurred for length with smaller individuals at ES ($11.9 \pm 0.8 \text{ cm}$, **Supplementary material 39**). Finally, density and length of *H. atra* were stable through time at PLA site (Permanovas, $df = 2$, $F = 0.311$, $p\text{-value} = 0.739$ and $df = 2$, $F = 0.967$, $P\text{-value} = 0.408$ respectively), with means of $2.9 \pm 0.9 \text{ ind.m}^2$ and $17.0 \pm 1.1 \text{ cm}$ respectively.

For ecological variables, spatial variability was detected only in organic matter load (**Table 13**), with the lowest value at ES ($1.95 \pm 0.05 \%$). Summer 2020 presented the lowest organic matter load ($2.26 \pm 0.06 \%$) whereas winter 2020 had the lowest percent of fine particles ($0.19 \pm 0.02 \%$). It should be noticed that results of PERMANOVAs indicated spatial variations for fine particles and chlorophyll a (**Table 13**), whereas two-by-two comparisons revealed no significant difference. The absence of significant spatial and temporal variability for fine particles and chlorophyll a was then the considered result.

Table 13: Two-ways PERMANOVA results for density and length of *Holothuria leucospilota* and *Stichopus chloronotus*, organic matter load, fine particle and chlorophyll a over sites and seasons.

	SS	Df	MS	F value	P-value
Density HI (N= 44)					
Station	35.99	4	9.00	10.32	< 0.001
Season	0.65	2	0.33	0.37	0.691
Residuals	32.27	37	0.87		
Length HI (N= 75)					
Station	754.10	4	188.53	4.83	0.002
Season	111.40	2	55.71	1.43	0.247
Residuals	2617.60	67	39.07		
Density Sc (N= 34)					
Station	1.71	2	0.86	2.29	0.119
Season	4.06	2	2.03	5.44	0.010
Residuals	10.82	29	0.37		
Length Sc (N= 45)					
Station	419.70	2	209.85	13.37	< 0.001
Season	18.60	2	9.32	0.59	0.557
Residuals	627.90	40	15.70		
Organic matter load (N= 69)					
Station	3.48	6	0.58	8.13	< 0.001
Season	1.06	2	0.53	7.61	0.001
Residuals	4.18	60	0.07		
Fines particles (N= 69)					
Station	2.15	6	0.36	6.07	< 0.001
Season	14.23	2	7.11	120.46	< 0.001
Residuals	3.54	60	0.06		
Chlorophyll a (N= 69)					
Station	33823	6	5637	3.01	0.012
Season	2384	2	1192	0.64	0.532
Residuals	112271	60	1871		

HI: *Holothuria leucospilota*; Sc: *Stichopus chloronotus*

SS: Sum of Square, Df: Degree of freedom, MS: Mean Square.

Biomarker patterns

Significant correlation was found between LLVY and AChE biomarkers ($r(80) = -0.257$; P-value < 0.019). Moreover, site, species, length of individuals and density were significant variables for both of these biomarkers (**Table 14**). Next sections present results involving these variables.

Table 14: Significance of individuals and ecological variables of each model (ANOVA results).

	SS	Df	MS	F value	P-value
AChE					
Rainfall_3months	9810	1	9810	0.89	0.347
Length	180842	1	180842	16.49	< 0.001
Density	154484	1	154484	14.09	< 0.001
Organic matter	123729	1	123729	11.28	0.001
Season	66410	2	33205	3.03	0.053
Species	329135	2	164568	15.01	< 0.001
Site	195119	6	32520	2.97	0.011
Length × Species	222803	2	111402	10.16	< 0.001
Rainfall_3months ×					
Site	232535	6	38756	3.53	0.003
Season × Species	402227	4	100557	9.17	< 0.001
Organic matter × Site	82460	4	20615	1.88	0.120
Residuals	1085675	99	10966		
LLVY					
Length	3.9797	1	3.98	16.8982	< 0.001
Density	9.789	1	9.79	41.5654	< 0.001
Species	6.3196	2	3.16	13.4169	< 0.001
Site	5.0126	6	0.84	3.5473	0.004
Length × Species	1.6608	2	0.83	3.5259	0.034
Residuals	17.8987	76	0.24		
AOPP					
Length	0.3541	1	0.35	6.9728	0.010
Organic matter	0.014	1	0.01	0.2755	0.601
Season	0.1537	2	0.08	1.5138	0.225
Species	2.3936	2	1.20	23.5686	< 0.001
Site	0.1198	6	0.02	0.3933	0.882
Season × Species	0.9396	4	0.23	4.6258	0.002
Organic matter × Site	0.5315	6	0.09	1.7444	0.118
Residuals	5.1287	101	0.05		

SS: Sum of Square, Df: Degree of freedom, MS: Mean Square.

(i) Spatio-temporal variations

The season variable was not significant in the three multiple linear models (**Table 14**), meaning that oxidative protein damage (LLVY/AOPPP) and neural response to toxicity (AChE) were constant through the time of the study. However, some spatial differences were

observed for LLVY and AChE (**Table 14**). Neural response to toxicity and the oxidative protein damage were higher at sites MNS, CAP, PTE and TE (**Figure 31**).

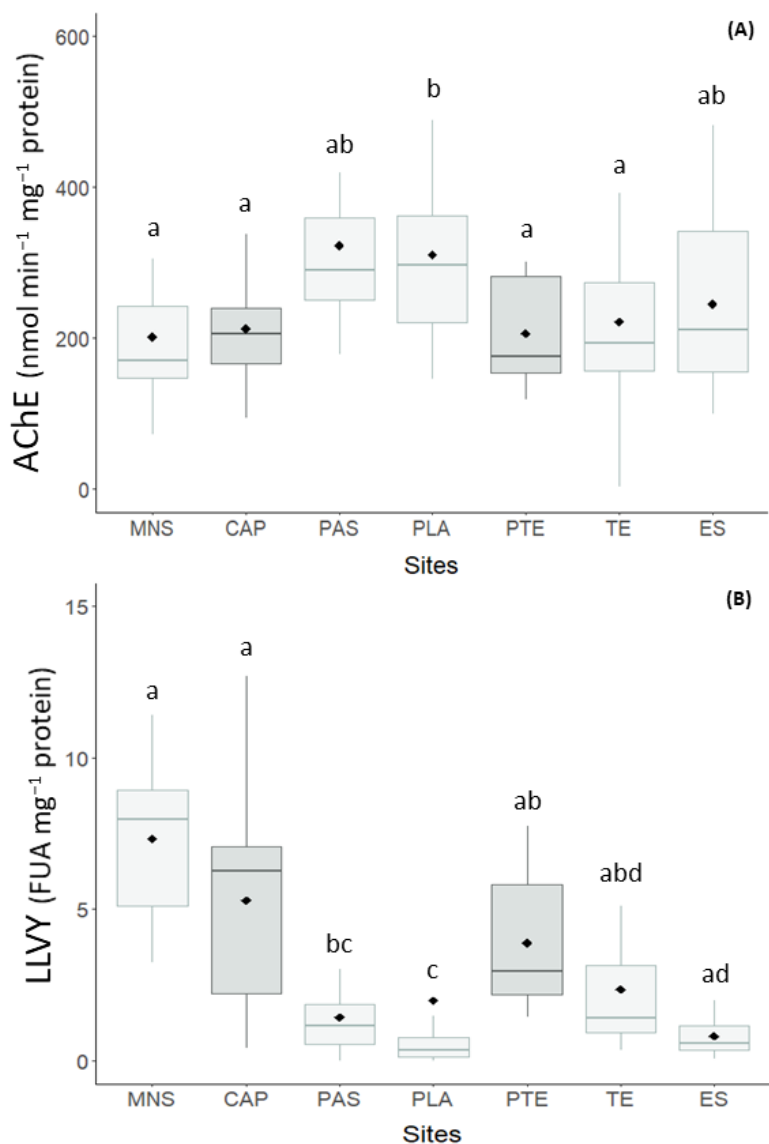


Figure 31: Spatial variability of biomarkers over the seven sites of the study. (A) Activity of acetylcholinesterase (nmol min⁻¹ mg⁻¹ protein) and (B) LLVY proteasome activity (fluorescence arbitrary units mg⁻¹ protein).

P -value < 0.05 are indicated by different letters.

Light gray: high density site. Dark gray: low density site.

MNS: Maître-Nageur-Sauveteurs, CAP: Cap-Méchant, PAS: Passe de l'Hermitage, PLA: Planch'Alizés, PTE: Petit Trou d'Eau, TE: Trou d'Eau, ES: Etang-Salé.

(ii) Specific variations

The three linear models found some species effect for biomarker variability (**Table 14**). Biomarkers of oxidative protein damage (LLVY/AOPP) and biomarker of neural response to toxicity (AChE) were congruent (**Figure 32**). Oxidative protein damage seems to be the most important in *H. leucospilota*, as the activity of LLVY (5.56 ± 0.77 fluorescence of arbitrary unit mg^{-1} protein) and the quantity of AOPP (0.04 ± 0.00 chloramine units mg^{-1} protein) recorded were higher in this species. Regarding AChE, *H. leucospilota* was the species presenting the highest neural response to toxicity (224.0 ± 11.5 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein) along with *Stichopus chloronotus*.

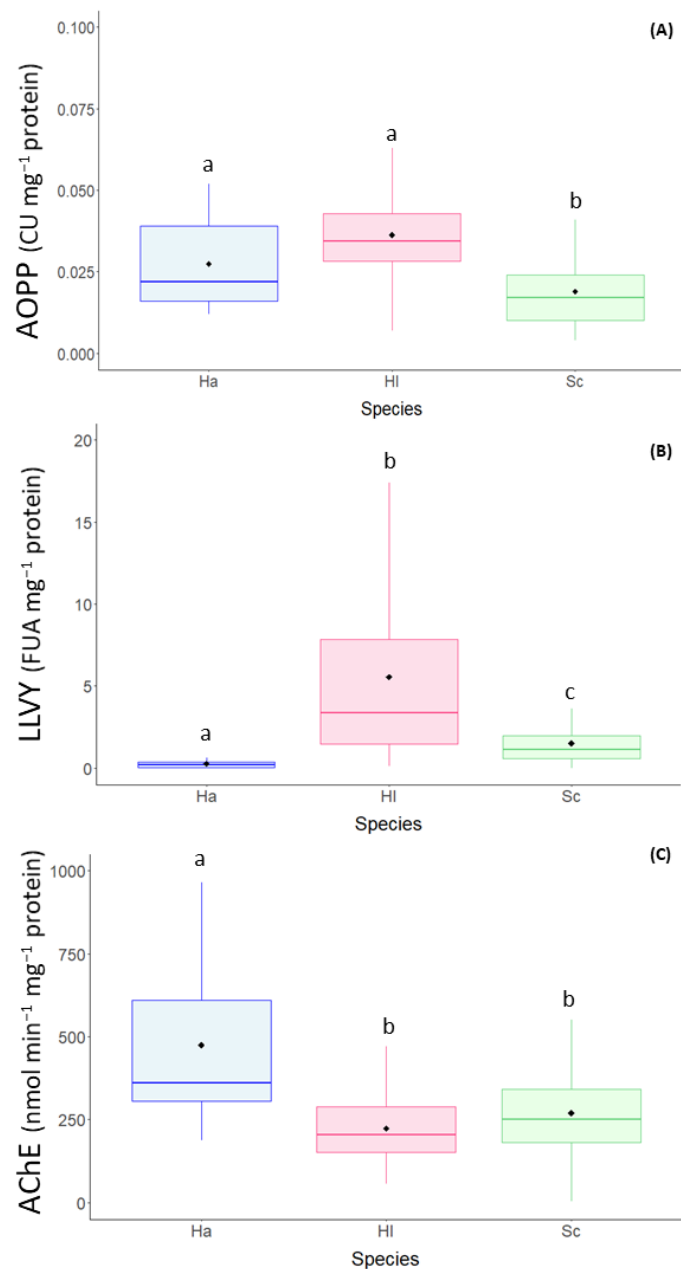


Figure 32: Specific variability of biomarkers. (A) Advanced oxidative protein product (chloramine units mg^{-1} protein). (B) LLVY proteasome activity (fluorescence arbitrary units mg^{-1} protein). (C) Activity of acetylcholinesterase ($\text{nmol min}^{-1} \text{mg}^{-1}$ protein) in muscles P -value < 0.05 are indicated by different letters.

Yellow: *Holothuria atra*, Pink: *H. leucospilota* and Green : *Stichopus chloronotus*

MNS: Maître-Nageur-Sauveteurs, CAP: Cap-Méchant, PAS: Passe de l'Hermitage, PLA: Planch'Alizés, PTE: Petit Trou d'Eau, TE: Trou d'Eau, ES: Etang-Salé.

(iii) Relation between population parameters and biomarkers

Oxidative protein damage (LLVY/AOPP) and neural response to toxicity (AChE) were correlated with length and density (**Supplementary material 40**). The activity of AChE in

muscles decreased with the length of individuals whereas it increased with the density, meaning that the longest individuals with the lowest density presented the highest neural response to toxicity (**Supplementary material 41**). Similar results were obtained for LLVY and AOPP (**Supplementary material 41**).

Discussion

Validation of the use of oxidative protein damage biomarkers as tool for monitoring organic pollution

For this current study, the efficiency of biomarkers of oxidative stress (AOPP and LLVY) was tested as an innovative tool to detect alteration of a large range of biological functions in wild sea cucumbers, thereby able to signal many environmental stressors. With no direct measurement of contaminants in sea cucumber tissues, the concomitant use of the AChE biomarker in this study aimed to corroborate the findings about the biomarkers of oxidative stress. The link between neural response to toxicity and oxidative stress is poorly studied. However, Gorokhova et al. (2013) have analysed the effect of exposure to contaminants on AChE, antioxidative enzymes (superoxide dismutase and catalase), and the oxygen radical absorbance capacity. They found a significant negative correlation between AChE and oxidative biomarkers, which possibly indicates mechanistic linkages between this neural response to toxicity and redox state regulation. Here, we evidenced the same strong negative correlation between biomarker of neural response to toxicity and oxidative protein damage. Physiological responses of organisms shown by inhibition of acetylcholinesterase and increase of oxidative protein and of proteasomal activity were congruent for spatial, temporal and specific patterns in Reunion Island (see sections below). AChE activity in wild sea cucumber populations of Reunion Island has revealed that they are subject to environmental stress due to organic pollution (Kolasinski et al 2010; our study). Consequently, oxidative protein damage biomarkers, appear to be reliable and efficient for assessing organic pollution and giving useful complementary information to describe the health of wild sea cucumber populations.

Specific, spatio-seasonal and pluriannual monitoring of neural response to toxicity and oxidative protein damage highlight the sensitivity of sea cucumbers to organic pollution

Data from Office de l'eau Réunion showed that organic pollutants such as atrazine and metolachlor or heavy metals such as lead and copper were present either in winter or summer 2020, in all protected reefs at Reunion Island. Unfortunately, methods currently used in Reunion Island do not allow to reliably quantify the concentration of organic pollutants or heavy metals in reefs, so only the presence/absence data can be used. However, even if validated quantification of such organic pollutants is not available in Réunion, temporal variations were expected for neural response to toxicity and oxidative protein damage studied here, as was hypothesized that pollution was partly related to rainwater runoff, which flows more intensely during austral summer in reef waters (Bigot *et al.*, 2000). This freshwater could have carried greater amounts of pollutants and fertilizers from agricultural uses or urban activity. Surprisingly, no temporal variations of the neural response to toxicity and oxidative protein damage, either intra seasonal or inter seasonal were significant, and no correlation was found between rainfall data and biomarker levels. Thus, the response of sea cucumbers to environmental stress was steady through seasons and even years.

Conversely, over long-term scale, Kolasinski *et al.* (2010) found that AChE reached 48.3 ± 15.7 nmol min⁻¹ mg protein⁻¹ in *H. leucospilota* and 11.0 ± 2.0 in *H. atra* in Reunion Island. These values are about 5 and 40 times lower than the values presented herein, meaning that environmental stress, including organic pollution, was higher at that time. Authors hypothesized that the environmental stress was supported by the use of organophosphates and pyrethroid insecticides due to the Chikungunya epidemic outbreak in April 2005. Threshold values are well-documented for AChE and so 20 to 30% inhibition compared to reference values, reflects an exposure to organic pollution (Escartin & Porte, 1996; Owen *et al.*, 2002), whereas 50% inhibition or more induces mortality (Barata, Solayan & Porte, 2004). Comparing results from Kolasinski *et al.*(2010) to our stable high values of AChE clearly highlights that: (1) sea cucumbers are sensitive to organic pollution as they can display a large range of AChE values, and (2) the level of organic pollution of reefs in 2020 was seemingly lower than in 2005.

Regarding oxidative protein damage, AOPP in sea cucumbers was only evaluated *in vitro* conditions thanks to experiments of exposure to different concentrations of pollutants.

Rabeh et al., (2020, 2019) and Telahigue et al. (2019) exposed different part of the body of *Holothuria forskali* (intestine, muscles and body wall, respectively) to different concentrations of mercury. They found that AOPP reached between 0.54 ± 0.08 and $1.30 \pm 0.19 \mu\text{mol.mg}^{-1}$ protein⁻¹, at the highest contaminant concentrations. Moreover, Telahigue et al. (2021) found AOPP reaching $0.30 \mu\text{mol.mg}^{-1}$ protein⁻¹ when respiratory tree of *H. forskali* were exposed to glyphosate. All these concentrations of AOPP are higher than those found in the body wall of wild sea cucumbers from our study. Surprisingly, even the non-treated individuals presented higher concentration of AOPP: 0.10 ± 0.01 to 0.59 ± 0.07 (Telahigue *et al.*, 2019, 2021; Rabeh *et al.*, 2019, 2020) compared to wild specimens from Reunion Island (maximum of $0.04 \pm 0.00 \mu\text{mol.ml}^{-1}$ protein⁻¹ for *H. leucospilota*). We can wonder if the difference in AOPP levels found between our results and other studies is more related to laboratory stress conditions and if the sea cucumbers used in the laboratory were really at a normal stress level. This comparison highlights the lack of reference threshold for studies carried out *in situ*.

Information about threshold values of AOPP and LLVY for effective stress are extremely limited. Based on the correlation with AChE levels, we can define our values of AOPP and LLVY as reference values for a mild stress in wild sea cucumbers. Tolerance, reversible physiological response and mortality threshold values need to be investigated in wet lab conditions. However, the cellular damage to the proteins recorded in this study, means that they have exceeded their regulatory capacities (Vasseur & Cossu-Leguille, 2003), but the inflexion point, leading to individual mortality, was not reached (Zhou *et al.*, 2021). Thresholds seem to be tissue, organ and species-dependant and no references were found for marine taxa.

Additionally, sea cucumbers appear to be good predictors of spatial distribution of organic pollution since variation of AChE activity and protein damage were recorded among sites during this study. Their ability to register small spatial variabilities at reef-scale in a context of a low organic pollution of reefs, again highlight their sensitivity. As methods used in Reunion Island are only efficient to detect the presence or absence of pesticides, this sensitive capacity of sea cucumbers could bring complementary information and help understanding the reef pollution.

Another the sensitive capacity of sea cucumbers about organic pollution, is the species-related pattern for biomarker response, with *H. leucospilota* the most stressed species. Sea cucumber species therefore have a differential sensitivity to environmental stress, as already recorded for mollusc species (Tim-Tim *et al.*, 2009) or fishes (Milošković *et al.*, 2016). Multi-species pollution monitoring could be relevant in such conditions of organic pollution. Many studies have used a multi-species approach to assess various types of pollution, such as microplastic, heavy metal (Milošković *et al.*, 2016), pharmaceutical (Damásio *et al.*, 2011) or pesticide pollution (Galassi *et al.*, 2008). The main argument in favour of using multi-species approach is to cover a wide range of feeding habits, which play an important role in the accumulation of pollutants in organisms. For sea cucumbers, *H. leucospilota* and *S. chloronotus* are surface deposit-feeders (Roberts *et al.*, 2000), they used their tentacles for searching food on the superficial sand layer, whereas *H. atra* is a sub-surface deposit-feeder (Roberts *et al.*, 2000), which introduce its tentacles up to 2 cm in sand to search food (Mangion *et al.*, 2004).

Healthy sea cucumber populations of Reunion Island reveal their resistance to organic pollution and their bioindication ability

Populations of sea cucumbers in Reunion Island have a patchy distribution with heterogeneous high densities, among the highest in the world (Conand & Mangion, 2002). Our results show that sea cucumbers in patches were more than one individual per meter square for each species, with up to 2.7 ± 0.3 , 2.9 ± 0.9 and 1.7 ± 0.2 ind.m² for *H. leucospilota*, *H. atra* and *S. chloronotus*, respectively. Such high densities have already been recorded previously (Conand *et al.*, 1998; Cuvillier *et al.*, 2016; Mangion *et al.*, 2004), meaning that populations of sea cucumbers in Reunion Island have been stable for few decades now. Two of the three high-density species in Reunion mainly reproduce asexually (Pierrat *et al.*, 2022b, Pierrat *et al.*, in submission), which theoretically contributes to higher population growth and indicates stable and favorable environmental conditions, thereby meaning populations may be in a good health. The stress level (shown in the previous section) is not reflected at the population scale, but only at individual level.

The high density and low and stable oxidative protein damage and neural response to toxicity assessed in this study, and the use of asexual reproduction by sea cucumbers, are congruent to hypothesize that sea cucumber populations of Reunion Island are healthy, and in consequence, resistant to organic pollution. In fact, the exposure response (AChE) and toxicity effects (AOPP and LLVY) recorded in individuals are low compared to other studies. This individual low stress is not transferred to the population level despite the toxicity by pesticides lasts for many years and the coral reef is subject to many other disturbing factors (REFS). Thus, some resistance mechanism is likely.

Finally, the ability for sea cucumbers to present a wide range of values for the three studied biomarkers (AChE, AOPP, LLVY) make them sensible indicators, whereas they are resistant to organic pollution, with good indicators at populational level. Both characteristics are very interesting to use them as bioindicator species to cover a wide spectrum of organic pollution levels.

Recommendations for future multi-biomarkers analyses on sea cucumbers

The lowest AChE activity and the highest oxidative protein damage were found in the two low-density sites, those with bigger individuals. This result was quite unexpected as many studies have significantly correlated the high density of sea cucumber and the stress, affecting different physiological and biological functions such as growth, feeding, endocrine response, energy budget (Xia *et al.*, 2017), or the crude protein or lipid contents in tissues (Dong *et al.*, 2010). Nevertheless, the negative correlation between AChE activity and length is well documented, and has already been reported for crustacean (Printes & Callaghan, 2003) or fish (Chandrasekara & Pathiratne, 2007). It is now well accepted that the negative correlation between AChE activity and individual length is linked to age (Rath & Misra, 1981; Jha & Rizvi, 2009; Haider *et al.*, 2014). Studies have shown that biomarkers of oxidative stress, such as AOPP, are also correlated with age, with an increase of oxidation and a decrease of enzyme activities (Cakatay *et al.*, 2003). However, the proxy between length and age should be used with caution with sea cucumbers because of the ability of some species to reproduce asexually by transversal fission (Dolmatov, 2014), meaning that one individual give two smaller

genetically identical individuals. These are therefore older than individuals of the same length resulting from sexual reproduction. Two of the three species reproduce asexually (*S. chloronotus* and *H. atra*; Pierrat et al., 2022b, in submission), but this length variation seems to have no effect on AChE activity and oxidative protein damage, regarding the results at high density sites. Thus, including a variable of clonality in future studies could highlight whether asexual reproduction influences the neural response to toxicity and the oxidative protein damage.

Conclusion

As far as we know, this study was the first use of biomarkers of oxidative protein damage to investigate environmental pollution in marine organisms. The increase of oxidative protein damage was strongly correlated with the inhibition of acetylcholinesterase, often used as biomarker of organic pollution. The two biomarkers of oxidative protein damage AOPP and LLVY also displayed the same temporal, spatial, species-dependant, and ecological responses as neural response to toxicity. That correlation made possible the validation of the use of AOPP and LLVY as efficient biomarkers for sea cucumbers. Therefore, we recommend a multi-biomarkers approach with the use of these two innovative biomarkers for assessing environmental stress, including organic pollution, in sea cucumbers and possibly in some other marine taxa. The results show that sea cucumbers are both sensitive and resistant to organic pollution in the steady environment found in Reunion coral reef, with a stress response limited to the individual level. Sea cucumbers can then be used as bioindicator species to monitor a wide spectrum of organic pollution levels. The correlation of the stress response to other environmental factors has to be further investigated. This method is a good tool that can help describing the global health of reef. This study, as a pioneer one, could be considered as reference for future assessments of pollution in wild sea cucumbers with oxidative protein damage.

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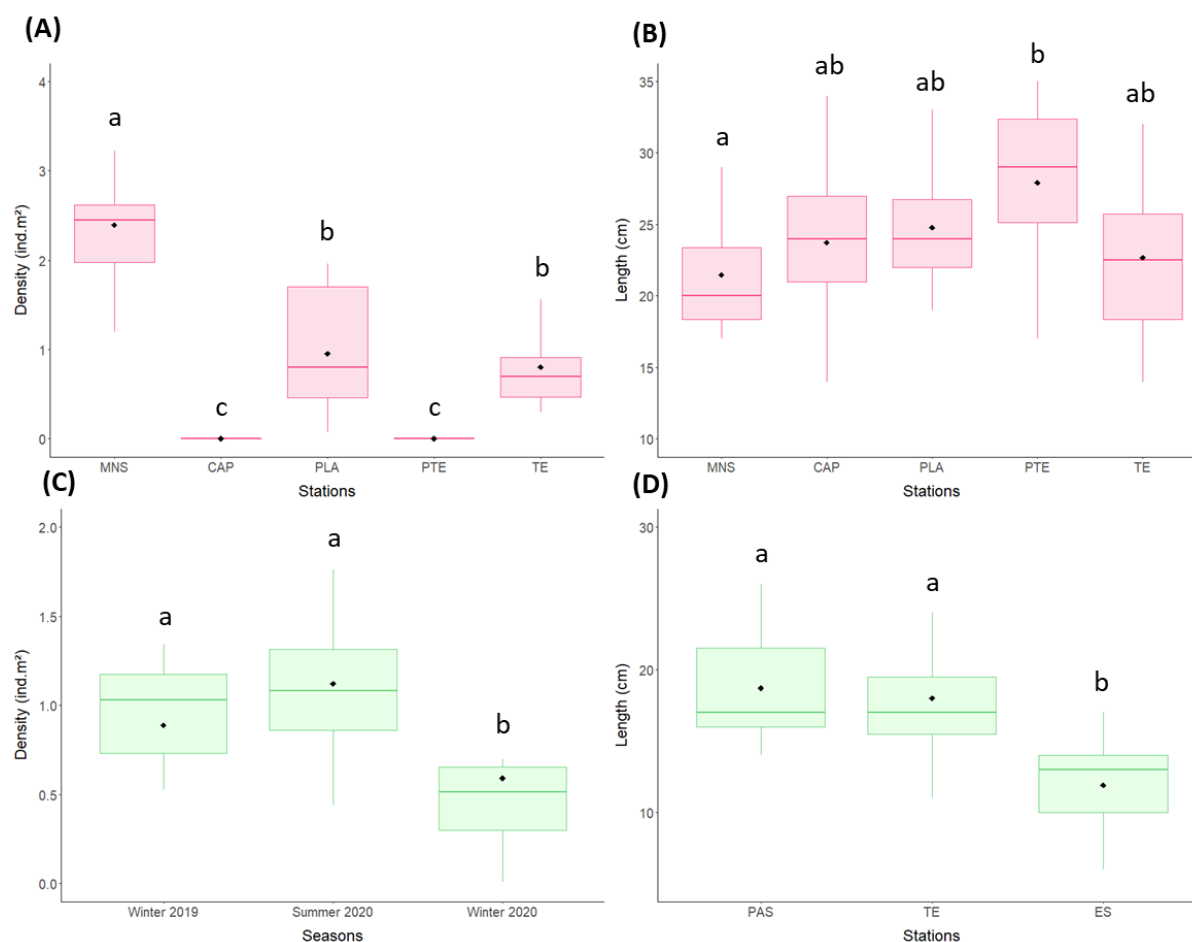
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Supplementary Material

Supplementary material 38: Final model of each biomarker (AChE, AOPP and LLVY).

Biomarker	Final Model	R²
AChE	<i>= rainfall accumulation + length + density + organic matter + season + species + site + rainfall accumulation × site + season species + length × species + organic matter × site</i>	0.6481
AOPP	<i>= length + organic matter + season + species + site + season × species + organic matter × site</i>	0.4677
LLVY	<i>= length + density + species + site + length × species</i>	0.5992

Supplementary material 39: Variability of individuals and ecological variables. (A) Mean density (ind.m² ± SE) of *Holothuria leucospilota* through sites. (B) and (D) Mean of length (cm ± SE) of *H. leucospilota* and *Stichopus chloronotus*, respectively, through sites. (C) Mean density (ind.m² ± SE) of *S. chloronotus* through seasons.



P-value < 0.05 are indicated by different letters.

Pink: *Holothuria leucospilota* and Green : *Stichopus chloronotus*

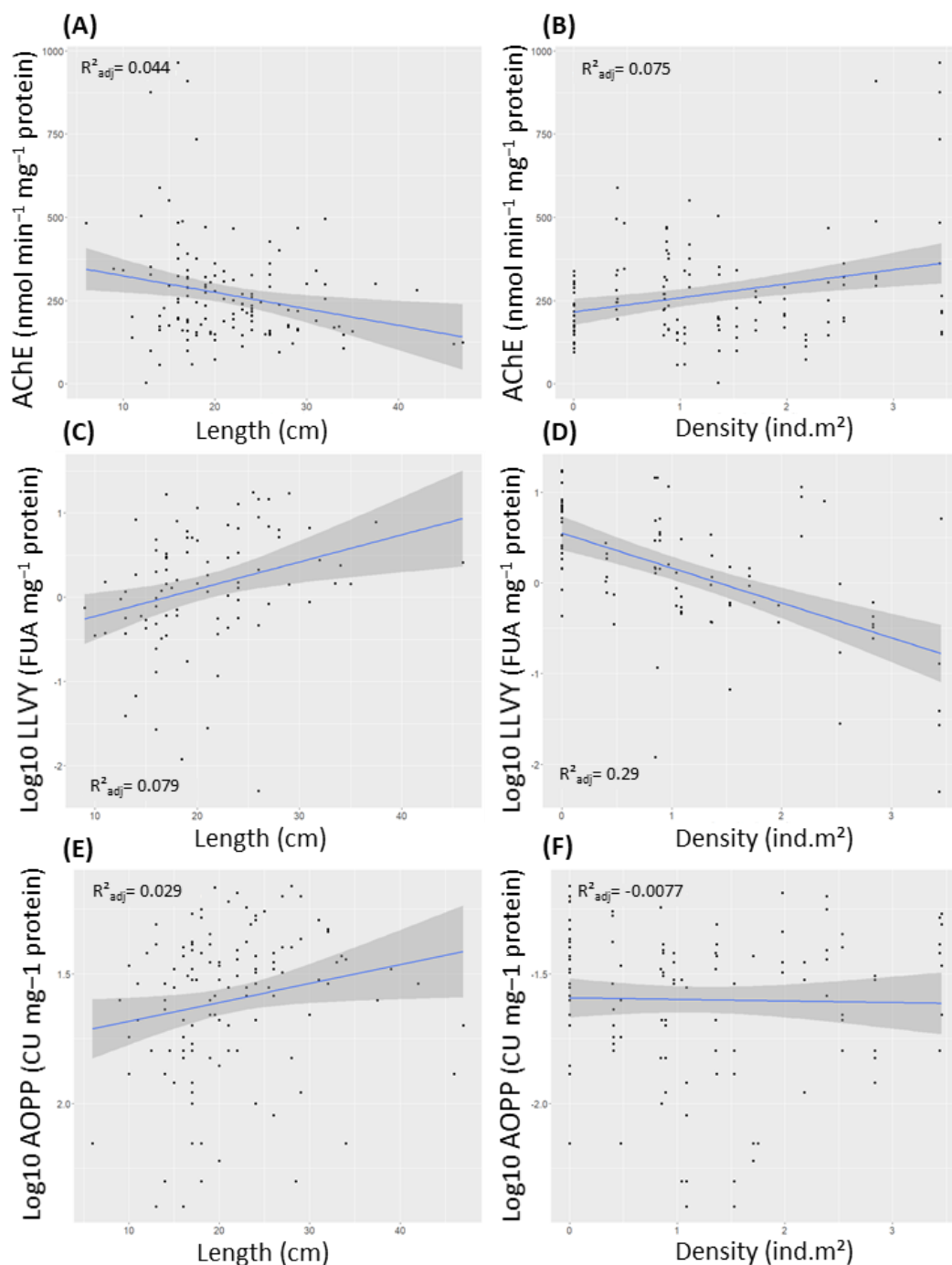
MNS: Maître-Nageur-Sauveteurs, CAP: Cap-Méchant, PAS: Passe de l'Hermitage, PLA: Planch'Alizés, PTE: Petit Trou d'Eau, TE: Trou d'Eau, ES: Etang-Salé.

Supplementary material 40: Pearson's correlation results for AChE, LLVY and AOPP with mean population length and density

	Length			Density		
	<i>r</i> value	Df	P-value	<i>r</i> value	Df	P-value
AChE	-0.227	128	0.009	0.286	128	< 0.001
LLVY	0.299	87	0.004	-0.550	87	< 0.001
AOPP	0.192	122	0.033	-0.023	122	0.801

Df: Degree of freedom.

Supplementary material 41: Correlation between, on the left: length of individuals (cm) and on the right: density of individuals (ind.m²) and (A) & (B) activity of acetylcholine esterase (nmol min⁻¹ mg⁻¹ protein); (C) & (D) LLVY proteasome activity (fluorescence arbitrary units mg⁻¹ protein); (E) & (F) advanced oxidative protein product (chloramine units mg⁻¹ protein).



Discussion générale

Cette étude a permis d'identifier plusieurs facteurs impliqués dans la distribution en agrégation de trois espèces d'holothuries tropicales à La Réunion, présentant de fortes densités ($> 1 \text{ ind.m}^2$). Plusieurs pistes de réflexion et d'amélioration peuvent être envisagées pour approfondir les connaissances sur ces facteurs d'agrégation et en identifier d'autres.

1. Facteurs d'agrégations des holothuries à La Réunion : dynamique spatiale et temporelle

Cette étude s'est intéressée à plusieurs types de facteurs (génétique, alimentaire, sédimentaire et anthropique) afin de comprendre les agrégations à forte densité observées chez trois espèces d'holothuries à La Réunion (*Holothuria atra*, *Holothuria leucospilota* et *Stichopus chloronotus*). La **Figure 33** représente l'ensemble de facteurs étudiés et leur influence sur la distribution des populations d'holothuries à La Réunion. Globalement, la structuration des populations d'holothuries communes est dominée par le facteur espèce, avec une dissociation entre les sites à faible et forte densité pour *H. leucospilota*. Les facteurs étudiés expliquent 45% de la variance observée.

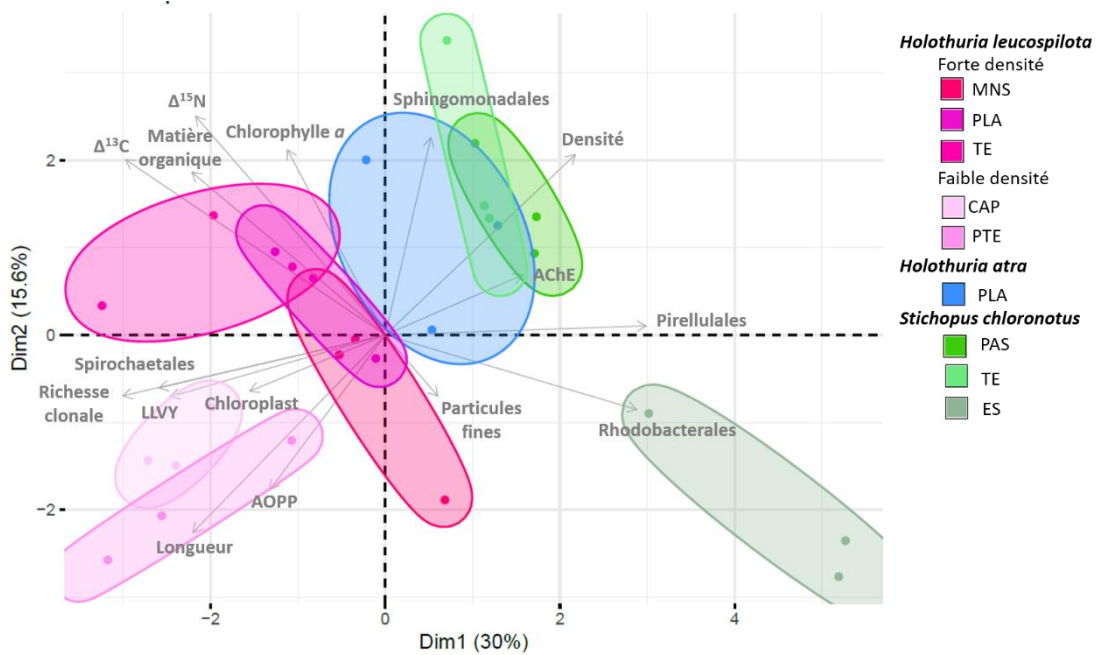


Figure 33: Influence des facteurs sur la distribution en agrégation à forte densité des trois espèces d'holothuries dominantes à La Réunion.

Facteur génétique : Richesse clonale (indice de 0 à 1 renseignant sur le part de reproduction asexuée). *Facteurs alimentaires* : Pirellulales, Rhodobacterales, Spirochaetales, Chloroplast, Sphingomonadales (contribution relative en % des principaux Ordres de bactéries assimilés). *Facteurs sédimentaires* : Matière organique (%), particules fines (%), chlorophylle *a* (mg.m²), $\Delta^{15}\text{N}$ et $\Delta^{13}\text{C}$ (‰). *Facteurs anthropiques* : AOPP (protéines oxydées, CU.mg⁻¹ de protéines), LLVY (activité chymotrypsine du protéasome, FUA.mg⁻¹ de protéines) et AChE (acétylcholinestérase, nmol min⁻¹ mg⁻¹ de protéines).

Holothuria leucospilota : en rose claire, faible densité (CAP et PTE) et rose foncée, forte densité (MNS, PLA, TE). *Holothuria atra* : en bleu (PLA). *Stichopus chloronotus* : en vert (PAS, TE, ES). Résultat d'une analyse en composante principale orthonormée. Les points d'une même couleur représentent les trois saisons d'échantillonnages (hiver 2019, été 2020 et hiver 2020).

(1) *Holothuria leucospilota* : populations stables à forte densité

Depuis les années 90, la dynamique spatiale et temporelle de *H. leucospilota* semble avoir évolué de manière positive, avec trois zones d'agrégations à forte densité réparties le long d'un gradient nord/sud dans le récif de L'Hermitage/La Saline. Le suivi saisonnier réalisé

dans le cadre de cette étude démontre une stabilité de la dynamique des populations de cette espèce. Parmi tous les facteurs sédimentaires suivis, seule une corrélation significative a été trouvée entre la présence de *H. leucospilota* et le taux de recouvrement en phanérogames, bien qu'elles soient présentes uniquement au nord du récif. En conséquence, l'augmentation des populations de *H. leucospilota* ne semble pas liée aux facteurs sédimentaires suivis et d'autres facteurs contrôlent cette dynamique.

Les analyses génétiques ont révélé que la reproduction asexuée par fission transversale ne se produit pas chez *H. leucospilota* alors qu'elle a cette capacité. Elle se reproduit donc principalement de manière sexuée, par émission des gamètes. En outre, les individus dans les agrégations à forte densité et ceux dispersés à faible densité dans le récif ne présentaient pas de différence génétique, suggérant des flux larvaires. Aucune information n'est disponible sur la distribution et l'abondance des juvéniles à La Réunion, qui ne sont pas observés dans les agrégations à forte densités. En effet, bien que des individus de petite taille ont été recensés lors de cette étude (23.7 ± 8.1 ; min= 3 ; max= 57), la distribution des tailles montre que leur présence est extrêmement anecdotique dans les zones de forte densité, malgré un effort d'échantillonnage robuste (près de 4000 individus mesurés lors de cette étude). Plusieurs études ont mis en évidence le comportement cryptique des juvéniles de plusieurs espèces n'occupant pas le même habitat que les adultes, *Parastichopus californicus* (Cameron & Fankboner, 1989), *Cucumaria frondosa* (Hamel & Mercier, 1996a) ou *Actinopyga echinites* (Wiedemeyer, 1992) et voir Shiell (2004) pour une revue. La distribution des juvéniles est attribuée à la dispersion larvaire et le comportement cryptique des juvéniles à la protection contre la prédation. Une fois atteint une taille suffisante, les juvéniles migrent vers les habitats des adultes (Young & Chia, 1982; Hamel & Mercier, 1996a), mais ces études n'expliquent pas les mécanismes en jeu. Le phénomène d'attraction conspécifique d'adultes pour la reproduction sexuée permettrait d'expliquer la stabilité des populations de *H. leucospilota* et l'absence de juvéniles dans les agrégations de forte densité.

Enfin, les caractères, généraliste sur le plan alimentaire et résistant au stress décelés chez *H. leucospilota* à partir des analyses du comportement alimentaire et du stress physiologique, soutiennent l'hypothèse d'attraction conspécifique pour la reproduction

maintenant ces populations stables. En effet, ces caractéristiques sont en faveur d'une bonne survie des juvéniles.

(2) *Holothuria atra* : population fluctuante à forte densité

Une seule zone d'agrégation à forte densité de *H. atra* est observée dans le récif de l'Hermitage/La Saline. La densité est élevée, près de 4 ind.m², bien qu'une légère diminution soit observée depuis 1990. L'analyse à échelle saisonnière n'a pas permis d'identifier une dynamique régulière, ce qui concorde avec l'absence de reproduction asexuée pour cette espèce. Une différence entre les longueurs moyennes entre saison chaude et froide serait alors observée, avec des individus plus petits en hiver lié à la reproduction asexuée.

Aucune relation entre le type de substrat et la dynamique de *H. atra* n'a été trouvée. Néanmoins, d'autres facteurs sédimentaires semblent favorables à l'agrégation de cette espèce. Il s'agit de la teneur en matière organique totale, la concentration en chlorophylle *a* et la teneur en $\Delta^{13}\text{C}$, qui présentent toutes les valeurs les plus élevées sur le site d'agrégation de *H. atra*. D'autres études ont également mis en évidence la présence de *H. atra* sur des sédiments riches en matière organique (Dissanayake & Stefansson, 2012) et en chlorophylle *a* (Viyakarn *et al.*, 2020). Ces facteurs sédimentaires sont signes d'une perturbation anthropique. En effet, la résurgence d'eaux terrestres dans le récif de La Saline induit un phénomène d'eutrophisation (Cuet *et al.*, 1988) et de pollution par les hydrocarbures aromatiques polycycliques (Tedetti *et al.*, 2011; Guigue *et al.*, 2015). Les fortes valeurs de chlorophylle *a* enregistrées sur le site d'agrégation de *H. atra*, concordent avec la composition du bactériobenthos : les Cyanobactéries sont les plus abondantes (32% du bactériobenthos) par rapport aux autres sites de l'étude. En conséquence, la présence de ces organismes photosynthétiques, favorisée par des perturbations anthropiques, semble influencer la dynamique spatiale de *H. atra*. Il apparaît alors primordial d'analyser les autres fractions du régime alimentaire de *H. atra*, notamment les microalgues. Sur le site de l'Etang Salé, l'absence de l'espèce pourrait être expliquée par une multitude de facteurs, y compris le type de substrat à dominance basaltique. Les résultats acquis ne permettent pas de conclure sur le sujet.

À l'image de *H. leucospilota*, aucun juvénile n'est observé dans la zone d'agrégation de *H. atra*. En atteste les longueurs individuelles mesurées (moyenne= 13.8 ± 4.8 cm ; min= 3 ; max= 33) et la distribution des classes de tailles observées pour cette espèce. Les observations d'adultes de *H. atra* ailleurs que dans la zone d'agrégation, sont rares. En conséquence, l'attraction conspécifique chez *H. atra* semble avoir lieu mais serait moins efficace que chez *H. leucospilota*.

(3) *Stichopus chloronotus* : populations en déclin

Les populations de *S. chloronotus* ont largement décliné depuis les années 90, et sont maintenant en forte densité (> 1 ind.m²) à Trou d'Eau uniquement. Cette baisse de densité est en concordance avec la diminution de la reproduction asexuée constatée par le biais de l'indice de la richesse clonale, de 0.09 en 2013, 0.10 en 2015 (Pirog *et al.*, 2017) à 0.24 en 2023 (Pierrat *et al.*, 2023). Cette espèce utilise donc les deux modes de reproduction, sexuée et asexuée, conduisant normalement à une augmentation de la densité, par attraction conspécifique pour la reproduction sexuée et par division des individus pour la reproduction asexuée. Des facteurs externes entraînent donc un déclin des populations de *S. chloronotus*. Aucun des facteurs sédimentaires étudiés n'est corrélé avec la dynamique temporelle de *S. chloronotus*. En outre, l'étude du comportement alimentaire a mis en évidence que cette espèce est spécialiste, comme précédemment montré dans l'étude de Uthicke & Karez (1999), ce qui peut la rendre plus vulnérable en cas de changements environnementaux. Les capacités d'adaptation de *S. chloronotus* face aux changements climatiques seraient limitées (Buccheri *et al.*, 2019), entraînant donc un déclin potentiel des populations dans un contexte de réchauffement global (Xu *et al.*, 2021). Les holothuries ne sont pas épargnées par les maladies. En effet, Han, Keesing & Liu (2016) ont répertorié 18 maladies possibles chez *Apostichopus chloronotus* sous conditions contrôlées, dont la majorité entraîne la mort des individus (Wang *et al.*, 2007; Zhang *et al.*, 2011). Des observations de pêche illégale d'holothuries dans les récifs de La Réunion ont été répertoriées sur les réseaux sociaux, sans informations sur les espèces concernées. La valeur commerciale plus élevée de *S. chloronotus* par rapport aux autres espèces en forte densité dans les récifs de La Réunion (Purcell *et al.*, 2018) et le fait que ses populations déclinent, laisseraient penser que les prises illégales seraient majoritairement

concentrées sur *S. chloronotus*. Selon Hernández-Flores *et al.* (2018), les populations d'holothuries présentant une distribution agrégée à forte densité seraient plus susceptibles d'être surpêchées due à la facilité d'accès à une grande quantité de ressources. De tels actes de pêches conduiraient très rapidement à l'épuisement des stocks (Price *et al.*, 2010; Hernández-Flores *et al.*, 2018). Renforcer la surveillance des zones protégées est à envisager, afin de stopper les prises illégales et préserver les populations d'holothuries tropicales de La Réunion. En outre, la prédation pourrait être un facteur régulant les populations d'holothuries. Francour (1997) a recensé 76 prédateurs d'holothuries, majoritairement des poissons, des étoiles de mer, des crustacés et des gastéropodes, pouvant localement réguler les populations. Kropp (1982) a estimé que *S. chloronotus* présentait un taux de survie inférieur à 50% face à l'attaque d'un gastéropode, *Tonna pernix*. Deux récentes études, dont une à La Réunion, font aussi état d'une prédation des holothuries par les tortues (Mulochau *et al.*, 2021; Papafitsoros, 2023). La prédation sur les juvéniles reste très peu étudiée, mais selon Francour (1997), elle serait l'un des facteurs clés régulant la dynamique des populations d'holothuries, en limitant l'immigration d'adultes dans les zones agrégées. La mortalité induite par ces facteurs hypothétiques (surpêche, prédation, maladie, changements globaux) n'est donc pas compensée par les deux modes de reproduction, conduisant ainsi au déclin des populations de *S. chloronotus*.

Pour l'ensemble des espèces, un large éventail de facteurs environnementaux régissant la distribution et la dynamique spatio-temporelle des holothuries a été mis en évidence dans la littérature scientifique, par exemple la température (Bonham & Held, 1963), l'hydrodynamisme (Barkai, 1991), la concentration en nitrite ou le pH (Asha *et al.*, 2015). Il s'agirait alors de coupler l'ensemble des facteurs environnementaux liés aux différents compartiments récifaux, sédimentaires, substrats durs et colonne d'eau, afin d'obtenir une vision globale de la dynamique récifale, gouvernant la distribution des espèces d'holothuries.

(4) Amélioration de la compréhension de la dynamique spatio-temporelle des holothuries

La perspective d'étudier d'autres facteurs environnementaux permettrait donc de mieux comprendre les phénomènes d'agrégations observés et leurs influences sur la dynamique spatio-temporelle des populations d'holothuries. En parallèle, il s'agit de mieux caractériser cette dynamique des populations d'holothuries. L'évolution des techniques de marquage des individus est lente pour les holothuries. En effet, grâce à leur forte capacité d'autolyse, les holothuries rejettent la plupart des marqueurs et régénèrent leur tégument au bout de plusieurs heures, jours ou semaines. La pérennité de la gravure de numéro d'identification, sur le tégument dorsal à une profondeur oscillant entre un et deux millimètres pour faire ressortir la chair blanche sous-jacente, a été testée chez *Holothuria whitmaei* (Shiell, 2006). Cette méthode de marquage induit des effets indésirables sur le comportement des individus jusqu'à plusieurs jours et ne permet de suivre les individus qu'environ trois semaines (Shiell, 2006). Rodríguez-Barreras, Serrano-Torres & Macías-Reyes (2014) ont mis en évidence que les méthodes de marquages externe par T-bar et interne par PIT (Passive Integrated Transponder) ont induit près de 60% et 40% d'éviscération, respectivement, chez *Holothuria mexicana* après moins cinq minutes. Le taux de rétention de ces deux types de marqueurs atteignait seulement dix semaines. Les marqueurs PIT ont néanmoins montré des résultats prometteurs chez *Cucumaria frondosa* avec un taux de rétention de 68% au bout de 300 jours (Gianasi *et al.*, 2015). La photo-identification, largement utilisée chez de nombreuses espèces, comme les requins (Holmberg, Norman & Arzoumanian, 2009), les baleines (Evans & Hammond, 2004), les tortues (Reisser *et al.*, 2008) ou encore les langoustes (Frisch & Hobbs, 2007), est une technique de marquage pérenne et non-invasive. La technique de capture-marquage-recapture des holothuries par photo-identification a déjà été expérimentée en milieu naturel, chez *Stichopus mollis* en Nouvelle-Zélande (Raj, 1998) et très récemment chez *Pearsonothuria graeffei* en Australie (Hammond & Purcell, 2023). Bien que prometteuses, les techniques sont encore au stade préliminaire, aucune automatisation n'est pour le moment développée et la reconnaissance des individus s'effectue manuellement. En outre, ces deux études ont été réalisées pour des populations d'holothuries peu dense. L'utilisation de la photo-identification pour les populations d'holothuries tropicales de La Réunion semble compromise. En effet, l'absence d'automatisation couplée à l'hyperdensité observée représenterait un travail titanesque pour

identifier les individus et trouver des critères discriminants, d'autant plus que les espèces d'holothuries dominantes à La Réunion sont unicolores, a priori sans motif particulier. Bien que *Holothuria atra* possède des ocelles sur le tégument dorsal, celles-ci sont formées de sable, donc possiblement instables. Les papilles de *Stichopus chloronotus* représentent, peut-être, le meilleur espoir pour utiliser la photo-identification chez les populations d'holothuries de La Réunion. Le marquage pérenne des individus contribuerait à la mesure de paramètres essentiels, tel que le taux de croissance et de mortalité, l'espérance de vie et l'âge de première reproduction, le poids du recrutement de juvéniles et d'adultes par immigration. Le développement de telles techniques remanierait la vision globale actuelle sur la dynamique des populations d'holothuries et représenteraient une avancée scientifique majeure dans ce domaine.

2. Les récifs coralliens de La Réunion : un milieu soumis à des perturbations anthropiques

Depuis plusieurs décennies, les chercheurs constatent la dégradation des récifs coralliens de La Réunion, notamment à cause de perturbations d'origines anthropiques (Cuet *et al.*, 1988; Semple, 1997; Kolasinski *et al.*, 2010; Tedetti *et al.*, 2011; Guigue *et al.*, 2015). Bien que cette étude ne se soit pas concentrée sur la mise en évidence de l'anthropisation des récifs coralliens de La Réunion, elle a révélé plusieurs indices en ce sens.

(1) Dominance des cyanobactéries : phénomène d'eutrophisation

La diversité du bactériobenthos des récifs de La Réunion est composée en grande partie par les Cyanobactéries (22.9% pour tous les sites et toutes les saisons confondus). Aucune variabilité temporelle n'a été remarquée, variant entre 21.7% en hiver et 24.2% en été, tandis qu'une variabilité spatiale entre les trois récifs semble se dessiner, avec 18% du bactériobenthos composé de Cyanobactéries dans le récif de l'Hermitage, 23% pour le récif de Etang-Salé et 27% pour le récif de la Saline. La présence de Cyanobactéries dans les sédiments renseigne sur un état de dégradation avancé du récif. En effet, plusieurs études ont mis en lien la présence de Cyanobactéries dans les milieux lacustres, estuariens et récifaux avec le phénomène d'eutrophisation relatif à l'apport de nutriments d'origines anthropiques (Touchette *et al.*, 2007; Lürling *et al.*, 2017). L'analyse des communautés bactériennes

benthiques des îles Xisha (latitudes tropicales), situées à plus de 300 km du continent et inhabitées jusqu'en 2013 (donc pouvant être considérées comme non polluées), n'a pas révélé la présence de Cyanobactéries (Dong *et al.*, 2022). Rusch, Hannides & Gaidos (2009) ont trouvé le même résultat sur une île de l'archipel d'Hawaï. En outre, De Voogd *et al.* (2015) ont examiné le bactériobenthos sur un gradient d'îles distantes de Jakarta, et ont montré que les Cyanobactéries sont retrouvées, en faible proportion, uniquement dans l'île la plus proche, potentiellement soumise à une plus grande pollution. L'eutrophisation des récifs de La Réunion, et notamment celui de la Saline a déjà été démontré depuis plus de deux décennies (Cuet *et al.*, 1988), avec l'apport de nutriments via les rejets d'eaux souterraines (Join, 1991). Les résultats de cette étude concernant la distribution et composition du bactériobenthos semblent concorder avec l'eutrophisation constatée.

(2) Augmentation des algues au détriment des coraux

Les récifs coralliens de La Réunion monitorés pendant cette étude, sont dominés par des débris coralliens recouvert de turf (39.4%, moyenne tous sites et toutes saisons), au contraire le taux de recouvrement des organismes vivants érigés est faible (9.6%), d'autant plus que cette catégorie comprend 7.8% d'organismes bioconstructeurs, les coraux sans turf et 1.8% d'organismes bioérodeurs, les éponges. Le taux de recouvrement du corail vivant avec turf atteignait 4.5%. La présence de turf dans les récifs a donc été estimée à 44%. Une variabilité spatiale du turf (débris coralliens avec turf et corail vivant avec turf) a également été remarquée à fine échelle dans cette étude, avec un taux de recouvrement de 39% en hiver et 47% en été (toutes saisons confondues). Dès 1993, Naim (1993) remarquait la dominance du turf dans les communautés benthiques lors de la saison chaude, avec 19% des coraux morts, dont 16% par étouffement du turf leur poussant dessus. L'ensemble de ces estimations est un indicateur de la dégradation des récifs coralliens de La Réunion, considérés comme sérieusement menacés avec 50% de leur surface dégradée en 2008 (Ahamada *et al.*, 2008). A large échelle, Bajjouk *et al.* (2019) ont mené une étude sur l'évolution de la composition en substrats des récifs coralliens de La Réunion entre 2009 et 2015 par le biais de données hyper et multispectrales. Les auteurs ont montré une progression du turf de 78% et une diminution de 28% des coraux vivant sans turf et de 39% du corail vivant avec turf durant cette période.

Ahamada *et al.* (2008) rapporte également que le récif de la Saline est caractérisé par une forte dominance de coraux massifs opportunistes, comme *Montipora circumvallata*, *Porites rus* et *Psammocora contigua*. Ces études à large échelle, couplées à cette étude à fines échelles sont concordantes et mettent en lumière un changement de phase du récif avec une diminution importante de la couverture corallienne en quelques années, au profit de la couverture algale. Ces modifications de l'habitat peuvent impacter les populations d'holothuries, en agissant sur la qualité du substrat, de la matière organique, *etc.*

(3) Stress physiologique des holothuries : pollution du récif par les pesticides

Les valeurs de l'oxydation des protéines et de l'activité du protéasome et de l'acétylcholinestérase, mesurées dans les tissus des trois espèces d'holothuries, témoignent d'une pollution organique dans les récifs de La Réunion. Bien qu'aucune variabilité temporelle au cours des trois saisons de suivi n'ait été constatée, une variabilité spatiale a été mise en évidence, avec les individus du site PLA possédant le stress le plus bas. L'ensemble des autres sites, du récif de l'Hermitage/La Saline et d'Étang-Salé étaient homogènes. En outre, *H. leucospilota* est l'espèce pour laquelle l'activité du protéasome enregistrée était la plus forte, tandis que celle de l'acétylcholinestérase était la plus faible. Le patron inverse est observé chez *H. atra*. Ces deux espèces se retrouvent en forte densité sur le site de PLA. Ainsi, la différence spatiale détectée pour le site PLA semble davantage liée à une variabilité interspécifique.


Cette étude tend à confirmer donc l'état de dégradation avancée des récifs coralliens de La Réunion en lien avec des perturbations anthropiques, telles que l'eutrophisation, la surpêche des herbivores et la pollution par les pesticides.

Afin de mieux comprendre la relation entre pollution du récif et dynamique des populations d'holothuries, plusieurs perspectives peuvent être envisagées. En parallèle des différentes mesures de biomarqueurs (AChE, AOPP et LLVY) dans les tissus des individus, le niveau de pesticides pourrait y être directement mesuré. Plusieurs études ont établi un lien entre altération physiologique, cellulaire, ou enzymatique et niveau de pesticide dans les tissus de quelques taxa marins, comme les poissons (Gusso-Choueri *et al.*, 2015) ou les étoiles

de mer (Den Besten *et al.*, 2001). De telles relations n'ont, à ce jour, pas été mises en évidence pour les holothuries. Par conséquent, la mesure des pesticides dans les tissus des populations naturelles d'holothuries tropicales de La Réunion permettrait de mieux interpréter les résultats obtenus pour les trois biomarqueurs (AChE, AOPP et LLVY) en comparant ces valeurs de références avec la littérature existante sur le dosage des pesticides dans les tissus des holothuries (Martín *et al.*, 2017; León *et al.*, 2021). Bien que l'exposition aux pesticides ne semble pas être un facteur clé dans la distribution des populations d'holothuries tropicales à La Réunion (pas de patron distinct entre les gammes de valeurs des sites à faible et forte densité), d'autres types de pollution pourraient influencer les agrégations observées. En effet, les holothuries ne sont pas épargnées par la pollution par les métaux lourds (Warnau *et al.*, 2006; Telahigue *et al.*, 2019) et la pollution plastique (Graham & Thompson, 2009; Renzi *et al.*, 2018; Mohsen *et al.*, 2019; Lombardo *et al.*, 2022). En outre, les trois outils d'échantillonnages passifs des polluants dans les récifs de La Réunion, déployés par l'Office de l'eau Réunion, présentent actuellement des limites. La méthode POCIS (Polar Organic Chemical Integrative Samplers) nécessite une calibration complexe afin d'obtenir des mesures quantitatives (Morin *et al.*, 2012). La méthode SBSE (Stir-bar Sorptive Extraction) enregistre des polluants non-polaires mais requiert un traitement de préconcentration sans lequel la quantification est imprécise (Prieto *et al.*, 2010). La dernière méthode DGT (Diffusive Gradient in Thin-films) détecte les métaux lourds, mais son fonctionnement nécessite une membrane protectrice externe sujette à l'encrassement biologique (Ji, Challis & Brinkmann, 2022). La quantification des polluants dans les récifs de La Réunion n'est donc pas fiable actuellement et seules les données sur l'occurrence peuvent être utilisées. L'amélioration de ces méthodes permettrait de mieux appréhender le niveau de pollution du récif. Il serait également pertinent de s'intéresser à l'influence d'événements climatiques exceptionnels, tels que de fortes précipitations ou le passage d'un cyclone, sur le transport de polluants dans les récifs et leurs répercussions sur la dynamique des populations d'holothuries. De telles mesures permettraient d'étayer le rôle des holothuries en tant qu'espèce indicatrice.

3. Le régime alimentaire des holothuries : pistes pour l'aquaculture

L'un des facteurs clés régissant la distribution des espèces la qualité et la quantité des ressources alimentaires disponibles (Shimadzu *et al.*, 2013; Curtis *et al.*, 2015). Cette étude s'est concentrée sur la fraction bactérienne du régime alimentaire de trois espèces d'holothuries présentant une distribution hétérogène à La Réunion. Les communautés bactériennes benthiques présentaient de fortes variations temporelles et spatiales, ne permettant pas de dessiner des tendances générales, ni à l'échelle de la communauté de bactéries, ni à l'échelle des trois familles dominantes (Pirellulaceae, Rhodobacteraceae et Chloroplaste (cyanobactéries)). En conséquence, la distribution hétérogène des populations d'holothuries à La Réunion ne semble pas en lien avec la distribution des communautés de bactéries benthiques. Ainsi, d'autre fraction alimentaire, comme les microalgues ou la méiofaune, provenant également d'autres compartiments récifaux, comme les substrats durs ou les herbiers, pourraient être étudiés. En outre, bien que certains taxons bactériens soient assimilés par les trois espèces d'holothuries, de fortes variabilités subsistent. La connaissance du régime alimentaire d'une espèce est l'une des prérequis fondamentaux afin de pouvoir la domestiquer. L'appauvrissement mondial des stocks d'holothuries (Friedman *et al.*, 2011) est en partie lié à la forte demande sur le marché asiatique (Rahman *et al.*, 2015; Rahman & Yusoff, 2017). En conséquence, l'aquaculture d'holothuries s'est développée dans plusieurs régions du monde (Conand, 2018). D'après cette étude, il est donc difficile d'identifier une alimentation optimale, sur le plan des bactéries, dans une perspective d'aquaculture. La majorité des études portant sur l'alimentation des holothuries en milieu aquacole décrivent une alimentation dominée par les microalgues pour tous les stades, larvaires, juvéniles et adultes (Battaglione, 1999; Huiling *et al.*, 2004; Agudo, 2006; Al-Rashdi, Eeckhaut & Claereboudt, 2012; Rakotonjanahary *et al.*, 2016; Duy *et al.*, 2016; Cabacaba & Campo, 2019; Campo, Cabacaba & Cosmiano Jr, 2019; Hamel *et al.*, 2022). Un large éventail de microalgues est utilisé, dont principalement, *Chaetoceros calcitrans*, *C. gracilis*, *Isochrysis galbana*, *Pavlova sp.*, *Nitzschia acicularis* ou encore *Thalassiosira weissflogii*. En milieu naturel, *S. chloronotus* et *H. atra* semblent privilégier des zones riches en microalgues (Uthicke & Karez, 1999). L'étude des microalgues semble donc une perspective intéressante pour comprendre plus en détail la distribution des holothuries à La Réunion.

 **La Réunion**
et son récif frangeant

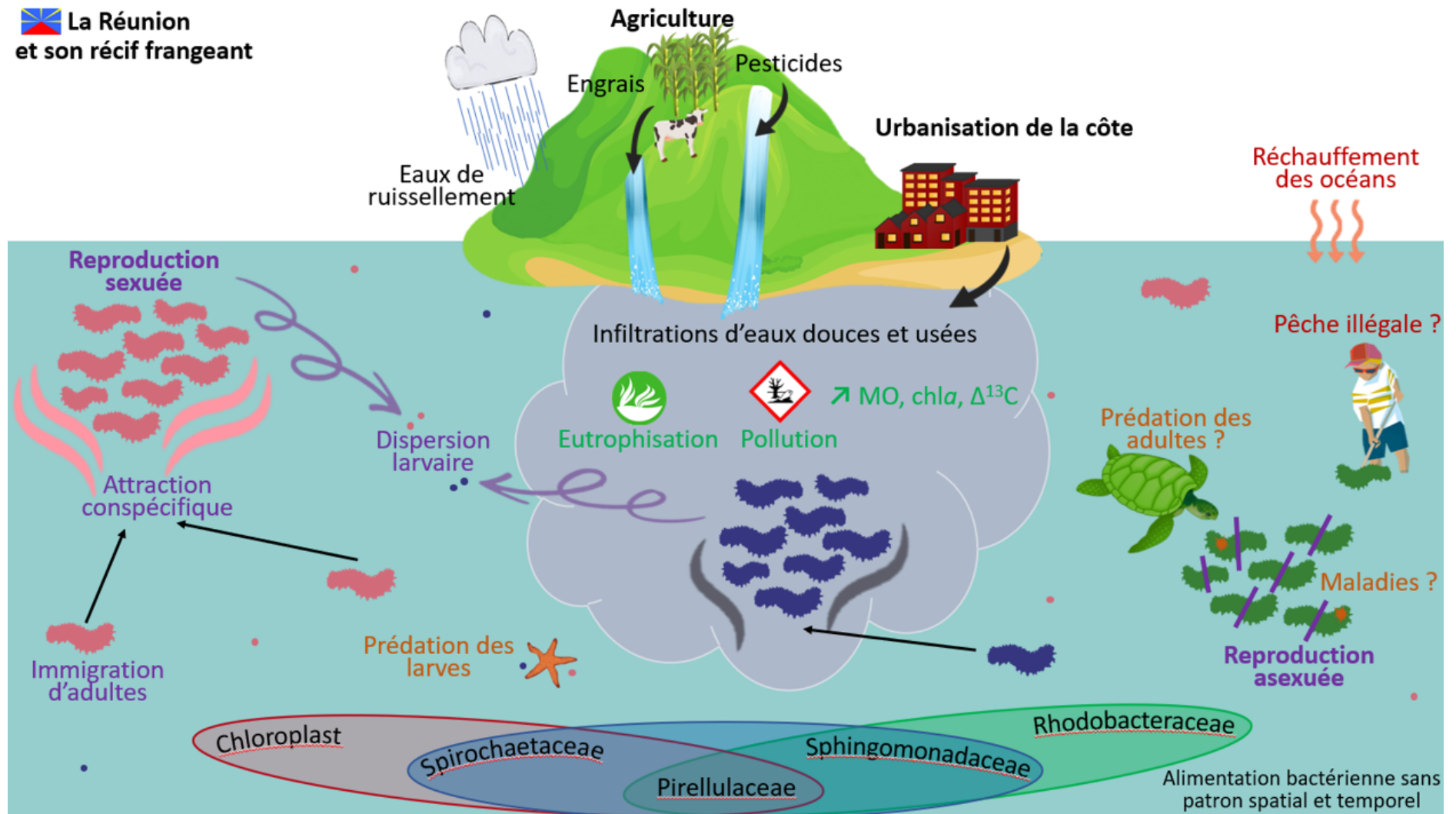


Schéma synthétique :

Facteurs influençant la dynamique spatio-temporelle des populations d'holothuries des récifs anthropisés de La Réunion.

En rouge : facteurs anthropiques défavorables à la dynamique des populations d'holothuries

En vert : facteurs anthropiques favorables à la dynamique des populations d'holothuries

En orange : facteurs naturels défavorables à la dynamique des populations d'holothuries

En violet : processus populationnels

Les holothuries :

Rose : *Holothuria leucospilota*

Bleu : *Holothuria atra*

Vert : *Stichopus chloronotus*

Conclusion

L'objectif principal de cette étude était d'identifier les différents facteurs influençant la distribution en agrégation des populations d'holothuries à forte densité ($> 1 \text{ ind.m}^2$) observées à La Réunion, dans un contexte récifal anthropisé. Les fortes densités d'holothuries sont généralement attribuées au mode de reproduction asexuée produisant par la fission transversale d'un individu, deux individus génétiquement identiques au stade adulte qui restent donc a priori à proximité de l'endroit de fission. Les analyses génétiques ont révélé que *S. chloronotus* se reproduisait de manière asexuée (richesse clonale = 0.24) tandis que, malgré leur présence en forte densité, *H. atra* et *H. leucospilota* se reproduisaient de manière sexuée (richesse clonale = 1). La structure génétique des populations d'*H. leucospilota*, à faible et forte densité, étaient similaires, suggérant des flux larvaires. En conséquence, d'autres facteurs régissent la distribution de ces deux espèces. Le suivi spatio-temporel, de la saison aux décennies, des populations d'holothuries a montré des tendances générales à la diminution d'abondance pour *H. atra* et *S. chloronotus* et seules les populations de *H. leucospilota* augmentent et prospèrent. Le déclin de *S. chloronotus* pourrait être lié à une diminution de la reproduction asexuée. La population de *H. atra* semble stable dans l'endroit du récif où la signature anthropique est la plus marquée (matière organique et $\Delta^{15}\text{N}$). Une faible corrélation a été mise en évidence entre la présence de phanérogames et de *H. leucospilota*. Le comportement alimentaire estimé par l'appréciation de la vitesse et du taux d'ingestion, indiquent un rythme diurne pour *S. chloronotus* contrairement aux deux autres espèces. En outre, *S. chloronotus* possède le plus fort taux d'ingestion et la plus grande mobilité, suggérant que cette espèce doit ingérer plus de sédiment et parcourir une plus grande distance pour subvenir à ces besoins nutritionnels. En conséquence, *S. chloronotus* serait donc spécialiste alors que les deux espèces d'*Holothuria* seraient généralistes. Bien que les Pirellulales est l'ordre le plus assimilé chez les trois espèces, la diversité du bactériobenthos assimilé diverge parmi elles. Les Shingomonadales et les Spirochaetales sont principalement assimilées par *H. atra*, les Spirochaetales et les Chloroplastes par *H. leucospilota*, et les Sphingomonadales et les Rhodobacterales par *S. chloronotus*. La distribution des holothuries n'est pas régie par la distribution spatiale et temporelle du bactériobenthos. Finalement, l'étude de nouveaux biomarqueurs attribués aux dommages cellulaires, en lien avec la mesure de la réponse neurale via l'acétylcholinestérase, a mis en évidence que le stress physiologique liée à une pollution par les pesticides est plus important chez *H. leucospilota*. Néanmoins, ce stress est inférieur à celui enregistré en 2005, indiquant que la pollution du récif par les pesticides semble avoir diminuée depuis 2005. *Holothuria leucospilota* semble être une espèce à la fois sensible et résistante, puisqu'elle est capable de produire de larges variations d'acétylcholinestérase tout en prospérant. En conclusion, la distribution en agrégation de *S. chloronotus* semble donc d'avantage liée à son mode de reproduction asexuée plutôt qu'à d'autres facteurs sédimentaires, alimentaires ou anthropiques. Le déclin de ses populations, et le fait que cette espèce soit considérée comme spécialiste, sont des indices alarmants concernant le devenir des populations de *S. chloronotus*. Les facteurs sédimentaires ne semblent pas avoir d'influence sur la distribution de *H. leucospilota*, tandis que *H. atra* prospère dans un habitat sous perturbation anthropique. L'étude d'autres facteurs environnementaux, notamment de la diversité en microalgues participant au régime alimentaire des holothuries, ainsi que l'étude d'autres compartiments récifaux, tel que la colonne d'eau et les substrats durs sont à envisager. En outre, l'attraction conspécifique mise en évidence très récemment chez les holothuries, pourrait expliquer en partie la distribution en agrégation observée à La Réunion. Des études sur ces mécanismes *in situ* sont à prévoir afin de comprendre la distribution de des populations d'holothuries de manière holistique.

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