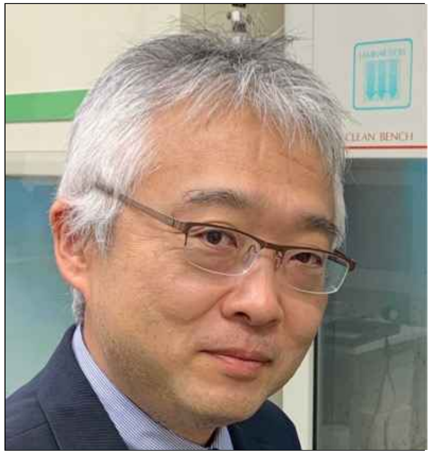
R & D of marine biodegradable plastics with degradation initiation switch function



PM：Ken-ichi Kasuya
Gunma Univ. Prof.
Organization: Gunma Univ, U Tokyo, Tokyo Tech, RIKEN, JAMSTEC

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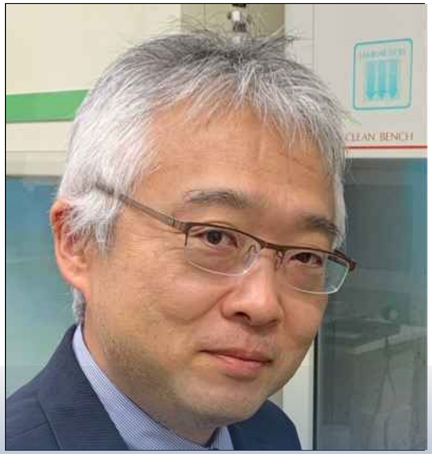

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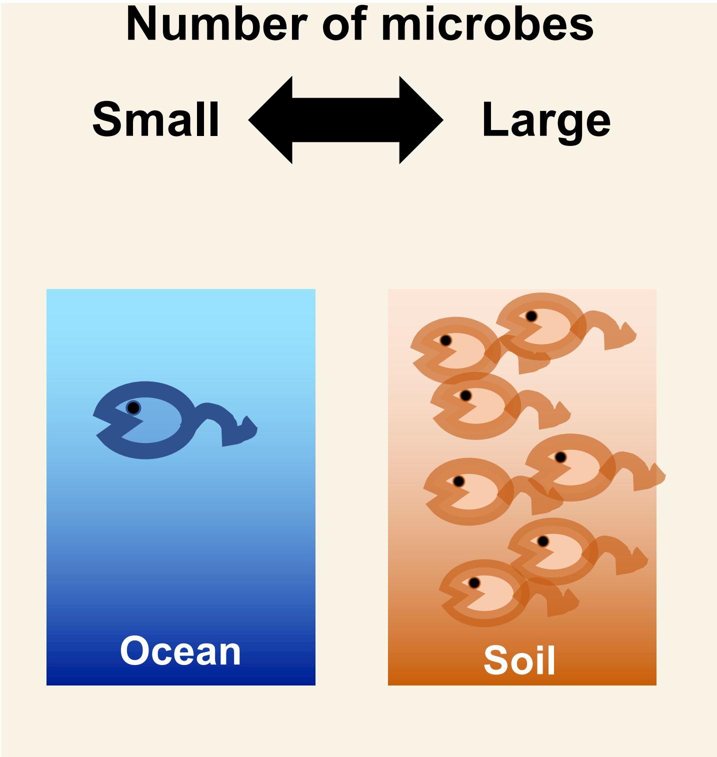
a. Background—Two challenges in marine biodegradable plastics



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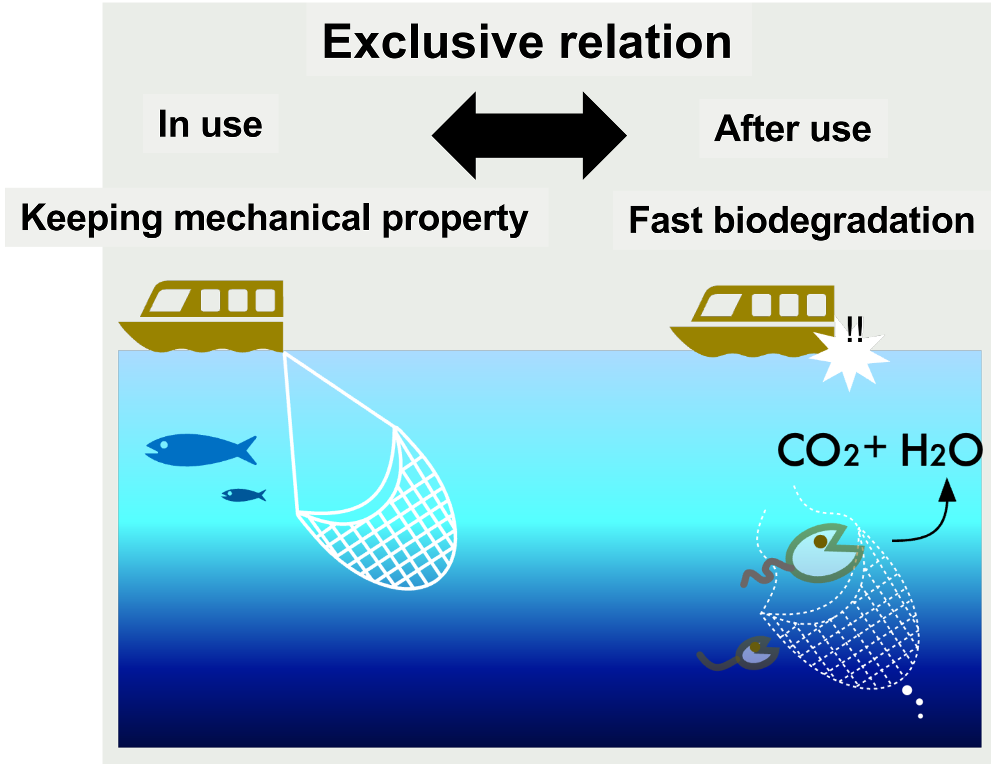
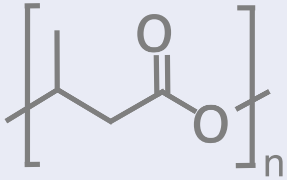
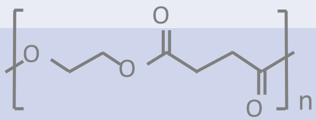
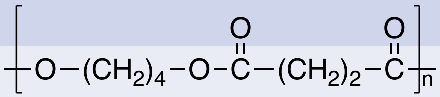
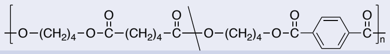
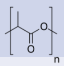
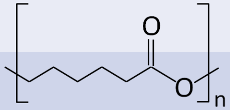


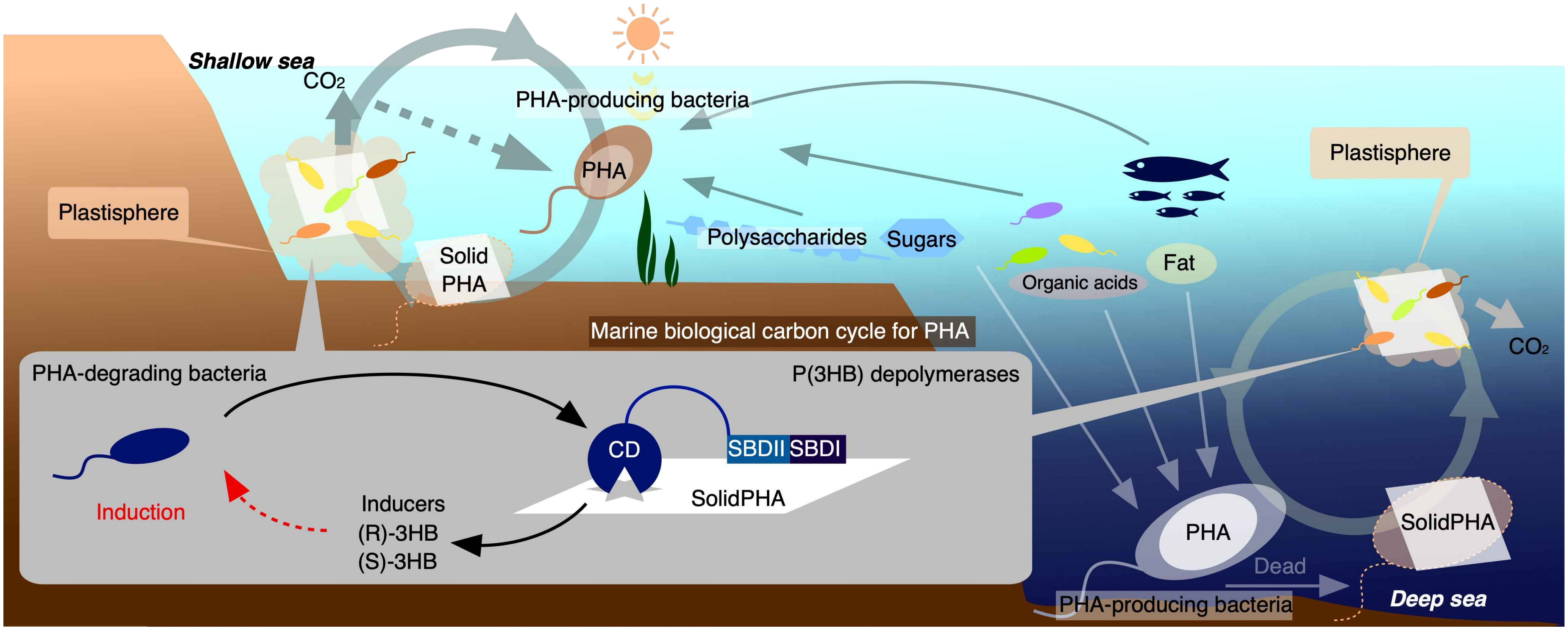
- The number of microbes in marine environment is small.
- Biodegradation of biodegradable plastics is very slow in marine environments.

- Biodegradable plastics are gradually biodegraded during use.

a. Background—Few marine biodegradable plastics

Polymers	Environmental degradability		
	Excellent	Depending on site	Poor
PHAs  Marine OK	Soil Freshwater Brackish water **Seawater** Aerobic sludge Anaerobic sludge Compost	-	-
PESu 	Soil Freshwater Compost Activated sludge	-	Seawater
PBSu  	Compost	Soil	Seawater Activated sludge Freshwater
PBAT 	Compost	Soil	Freshwater Seawater
PLA 	Compost	Soil	Seawater
PCL Marine OK	Soil Freshwater **Seawater** Compost	-	-

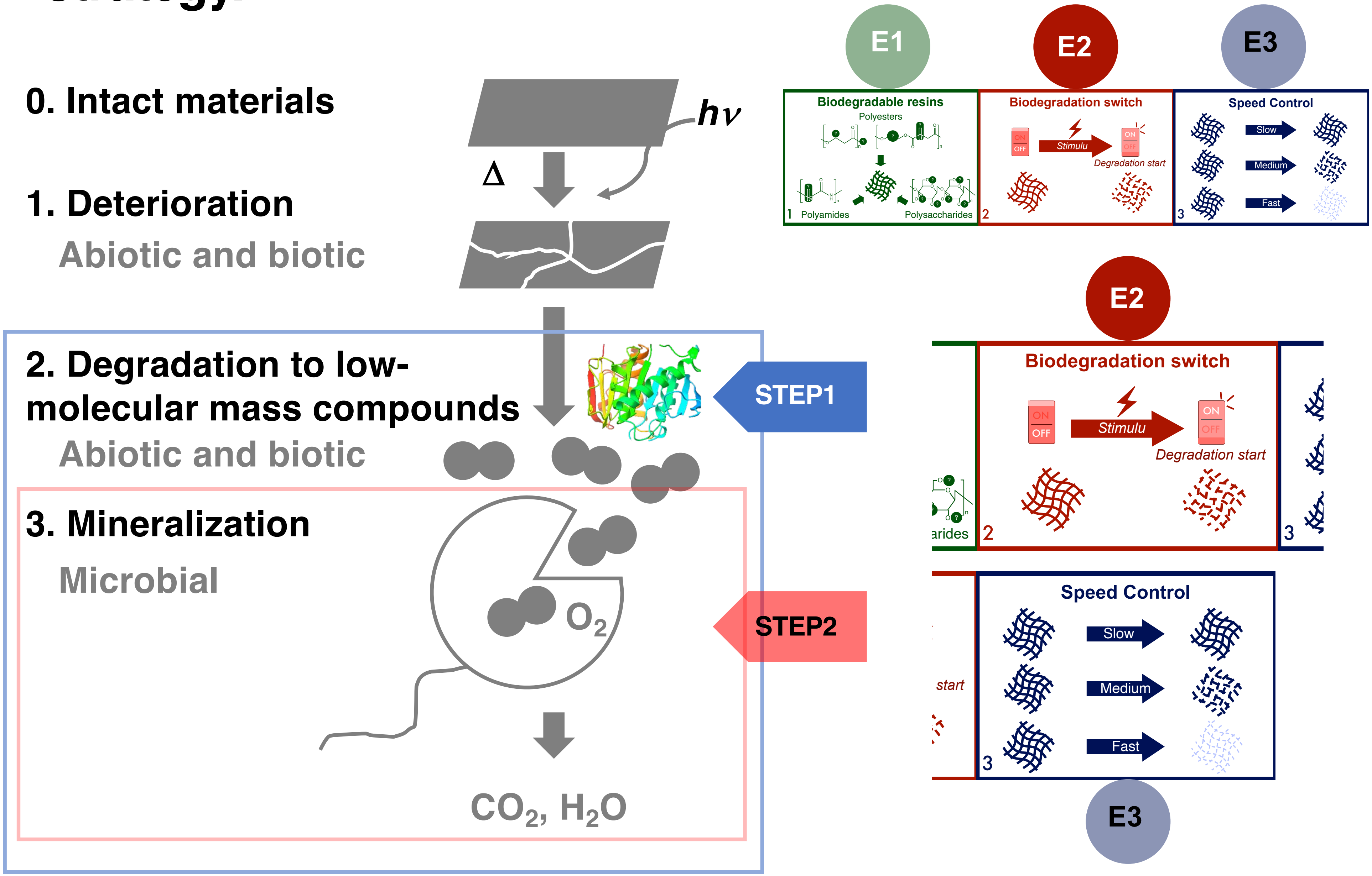
a. Background—PHAs are biodegraded in marine environments.



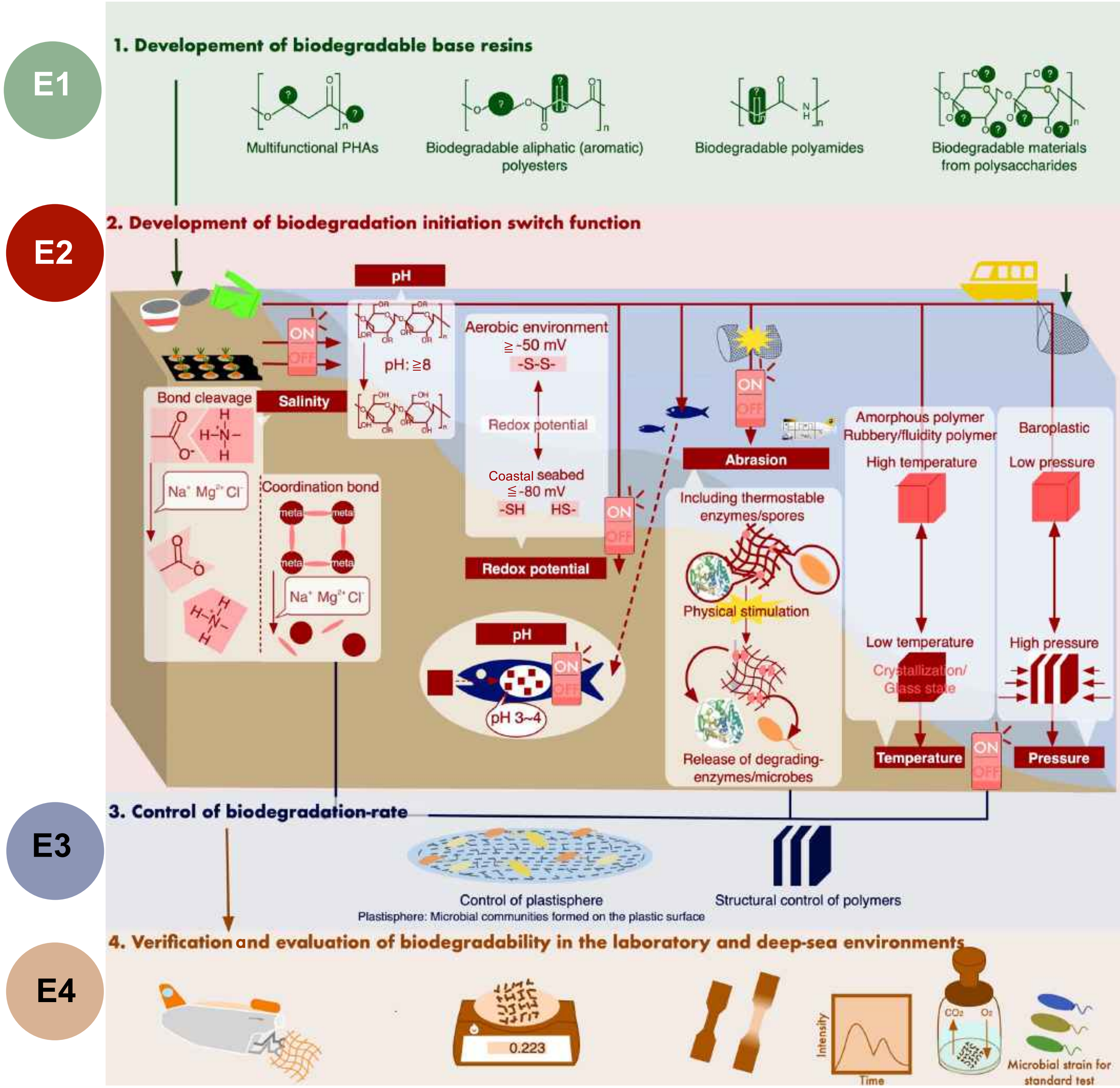
Polym J **53, 47–66 (2021)**

PHAs are produced and biodegraded in marine environments.
Biological carbon cycles for PHA exist in the marine environment.

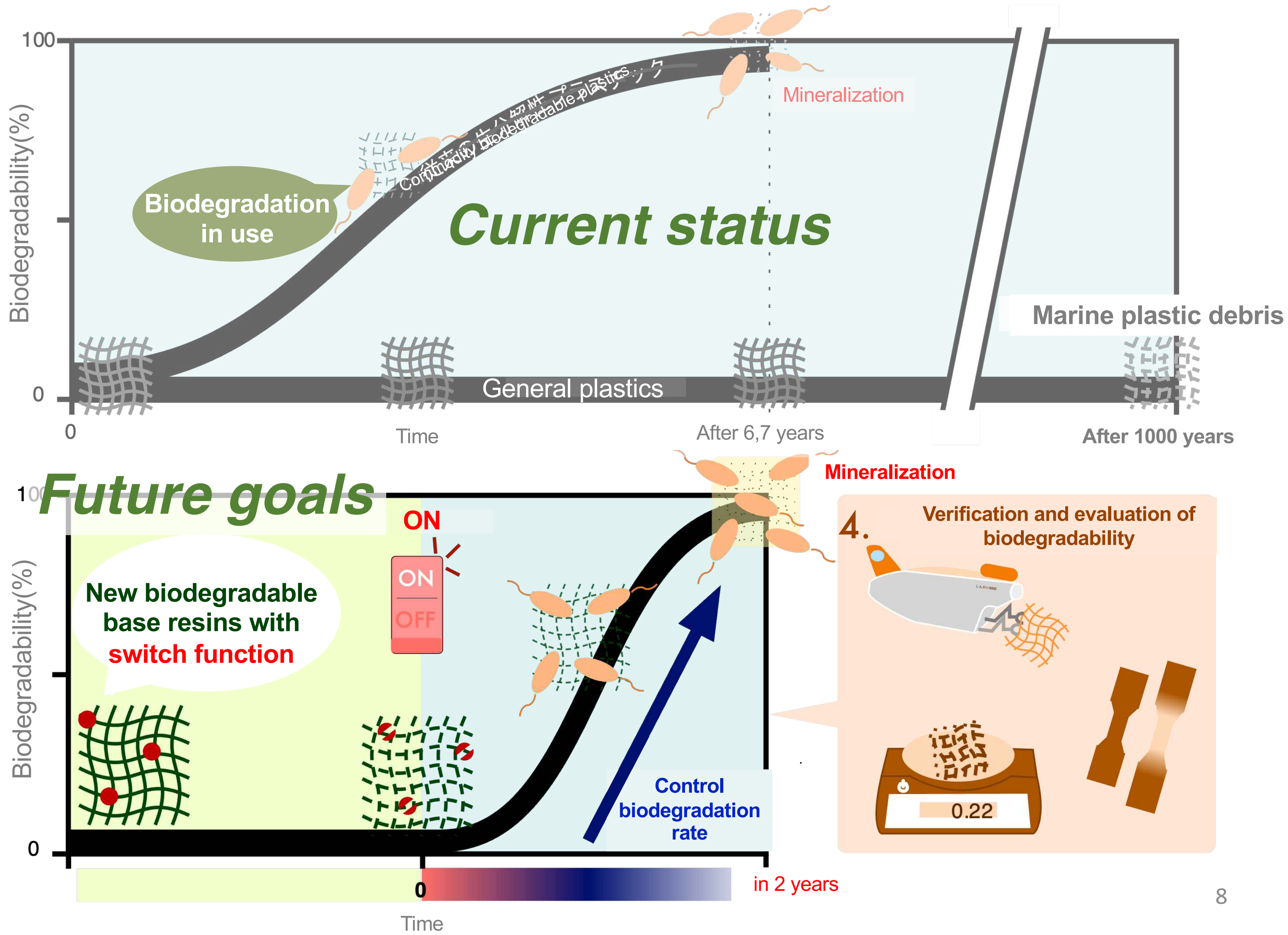
a. Background—Biodegradation mechanism and R & D strategy.



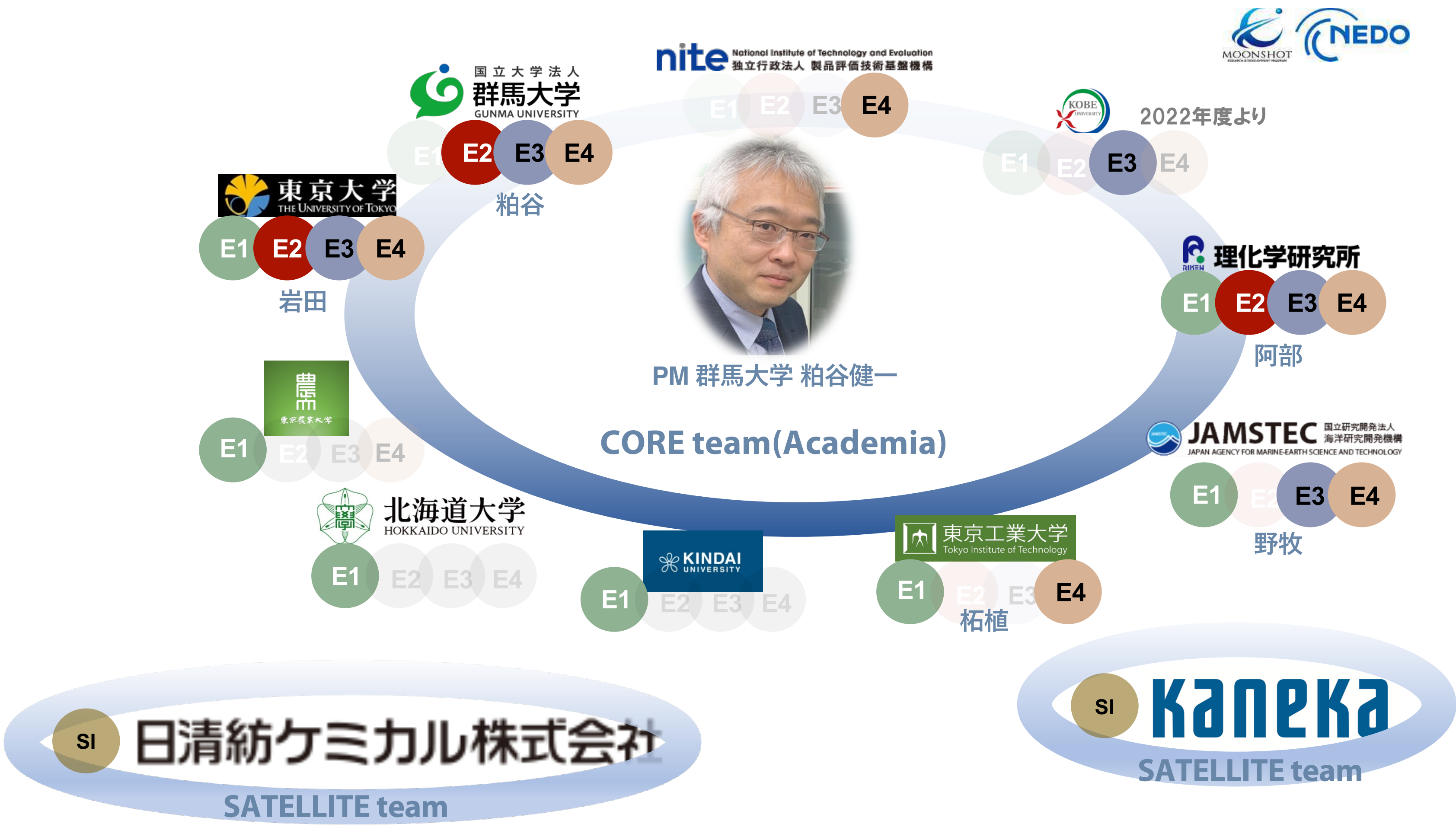
1. R&D items of the project（E1, E2, E3, E4）



2. Current status and future goals



3. Organization of R & D



SATELLITE teams (Companies and Academia)

Since 2022, academic-industrial satellite teams are formed to accelerate the social implementation of outcomes developed by the core team.

PM promotes the systemization of elemental technology, reorganizes the teams, and selects the themes to maximize the outcomes.

4. International cooperation and science and technology dialogue with the public

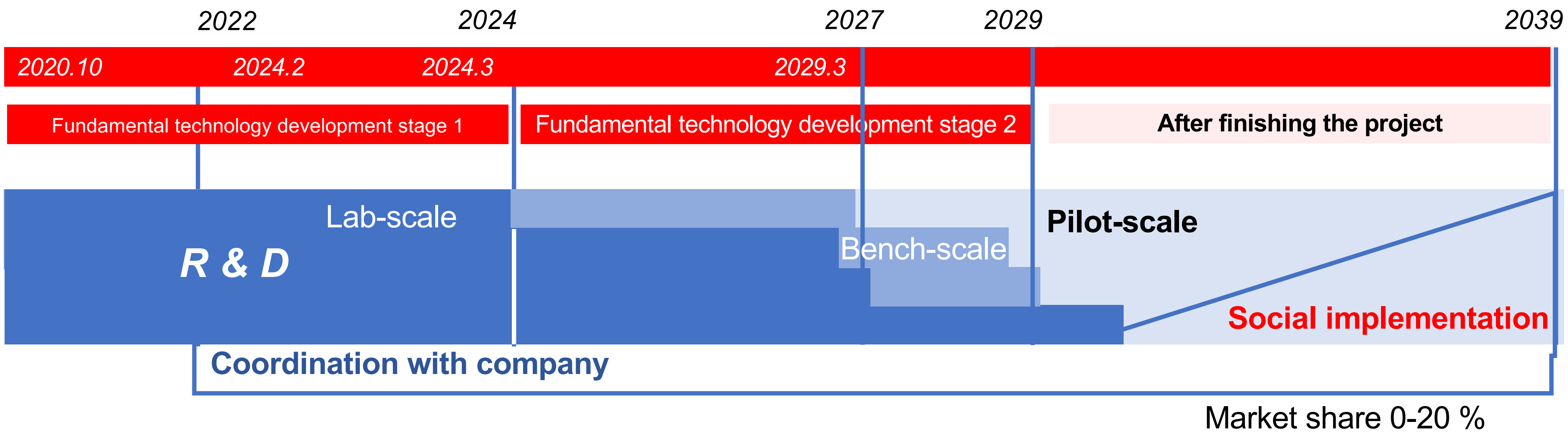
We evaluated degradability of biodegradable plastics in the ocean surface layer in cooperation with the National Oceanic and Atmospheric Administration (NOAA).

Buoys installed in the Great Pacific garbage patch is used as a test site for degradation of biodegradable plastics. When the buoys are replaced every year, biodegradable plastics are collected and newly installed. The degradability of biodegradable plastics in the marine surface environment is verified as part of international joint research.

As part of GIGA School x Deep Sea, a new biodegradable material was installed 855 m off Hatsushima Island with more than 24,000 elementary school students and the Minister of MEXT via a live online broadcast.

Above：The material before and after 4.5 months in deep-sea. Right：News site reporting the results of the material developed in this project and demonstrating on-site biodegradability.

5. Time schedule for R & D

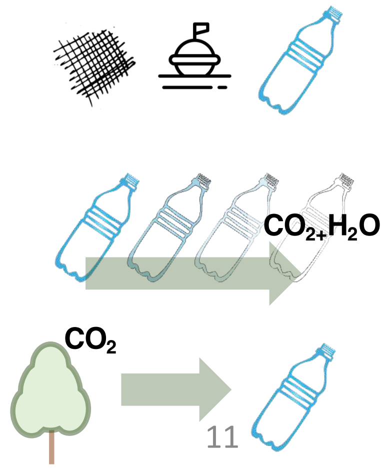


6. Goal of our project（2029）

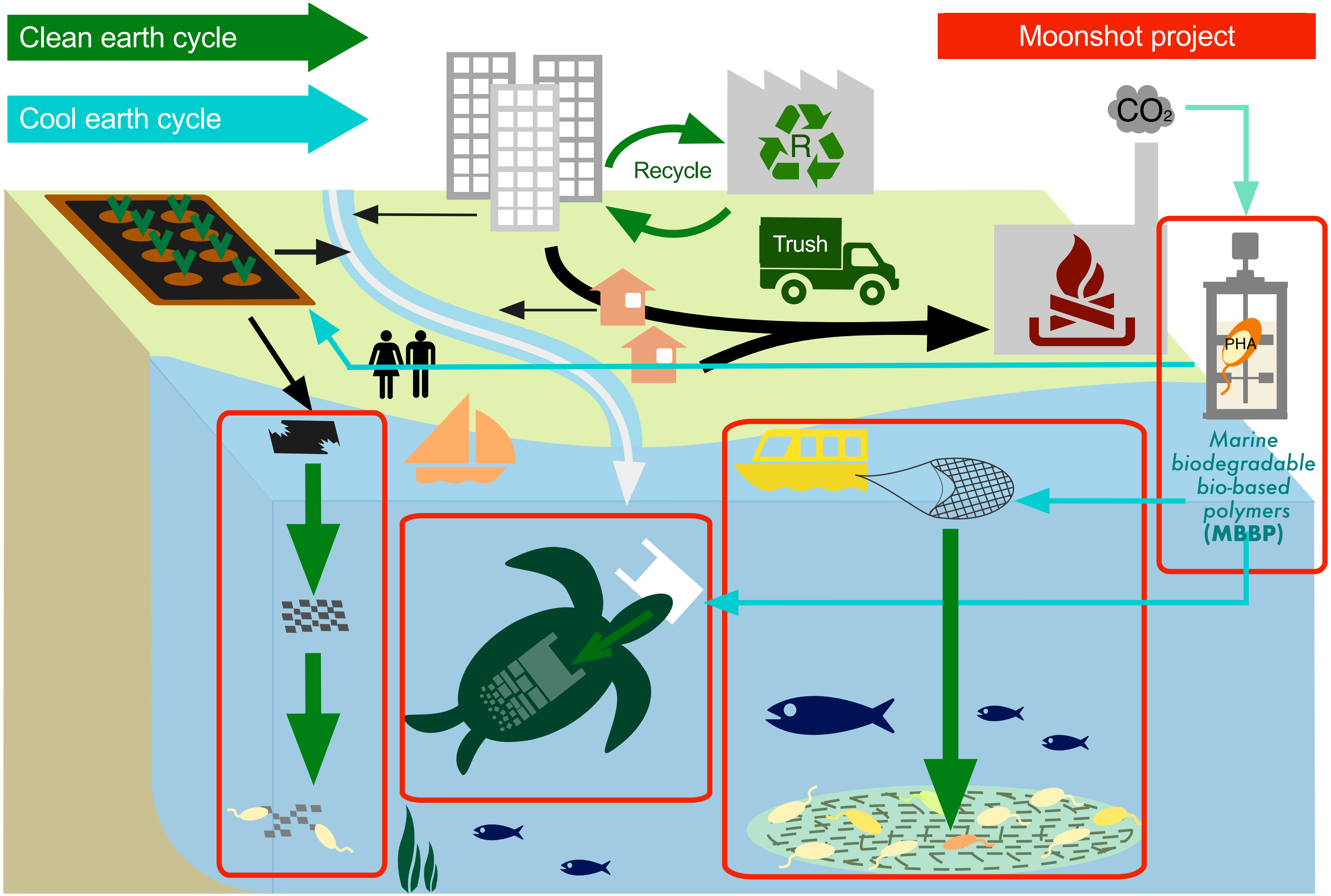
① We create three or more new marine biodegradable plastics that exhibit 90% biodegradability in seawater at 30 ˚C in 6 months after the switching function exerts.

② We demonstrate the biodegradability of these new marine biodegradable plastics having the switching function in marine environments, including deep sea.

③ We create new marine biodegradable base materials made from biomass and carbon dioxide.



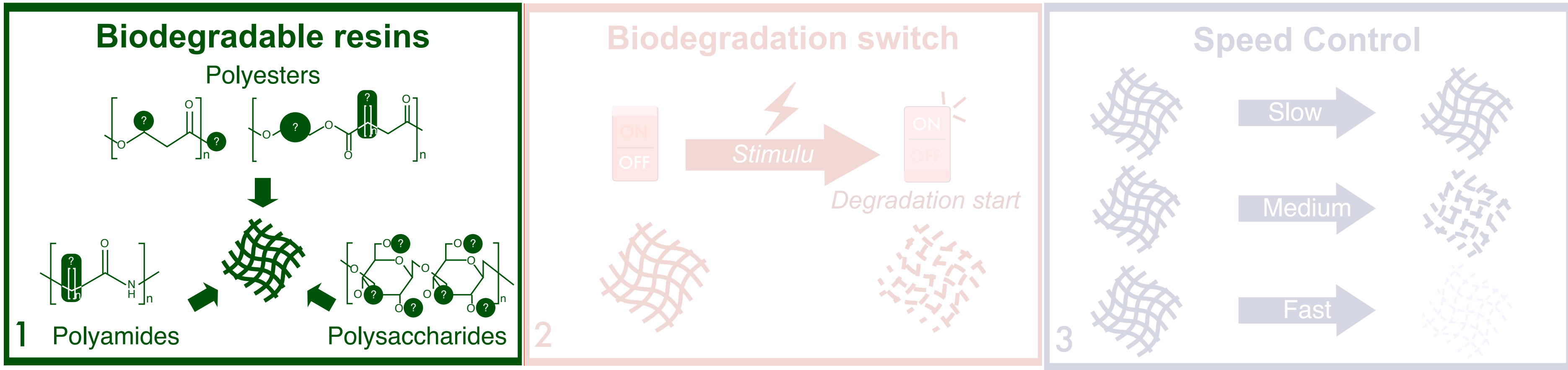
7. Social implementation



E1

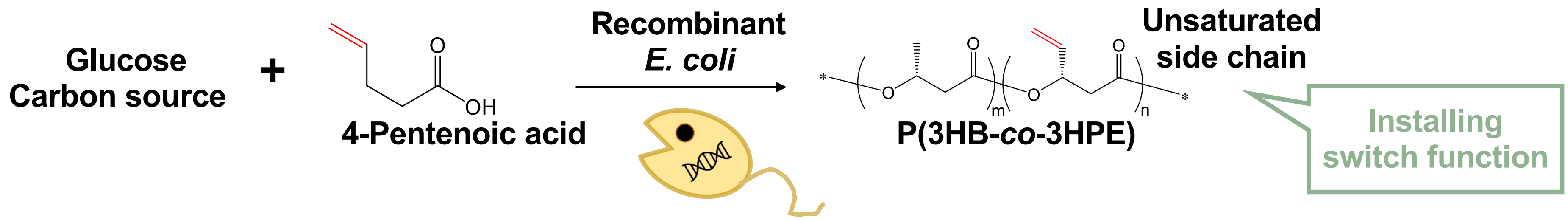
8. Major results regarding ongoing topics

E1, R & D of marine biodegradable base resins that can introduce switch function



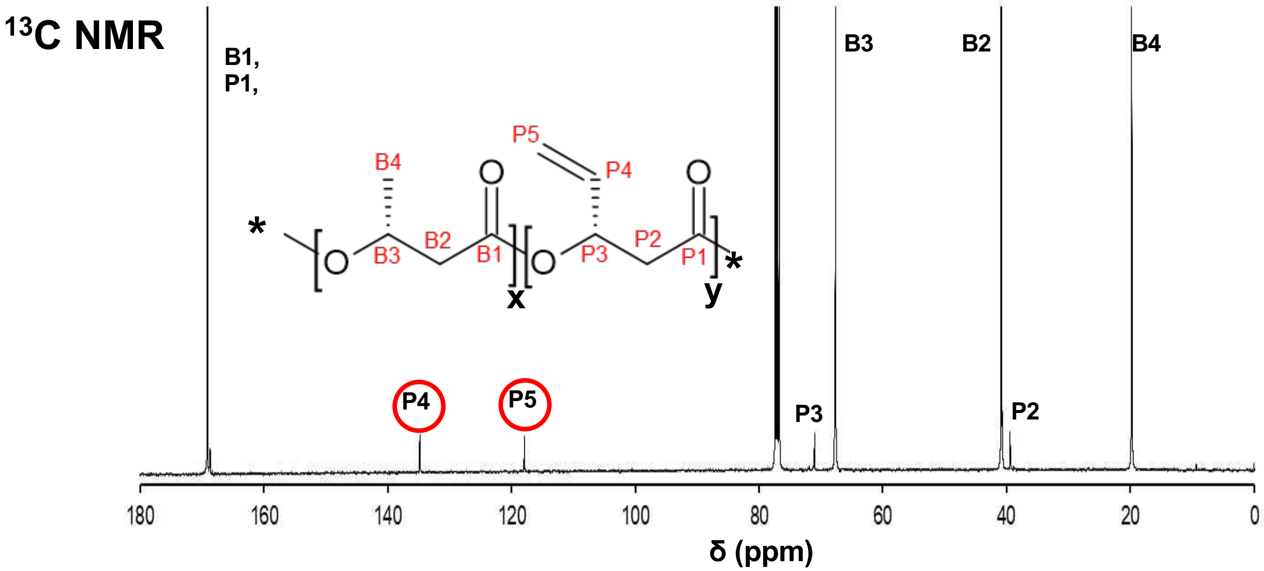
E1 :	R & D of marine biodegradable base resins	PHA	Functionalization Toughening Fixation of carbon dioxide
		Polysaccharide	Plasticization/Biodegradability Toughening
		Synthetic polymer	Biodegradable building-block polymer

E1 Base materials for installing switching functions



Culture Condition： LB medium + 4-Pentenoic acid + Glucose, 30˚C, 72 h

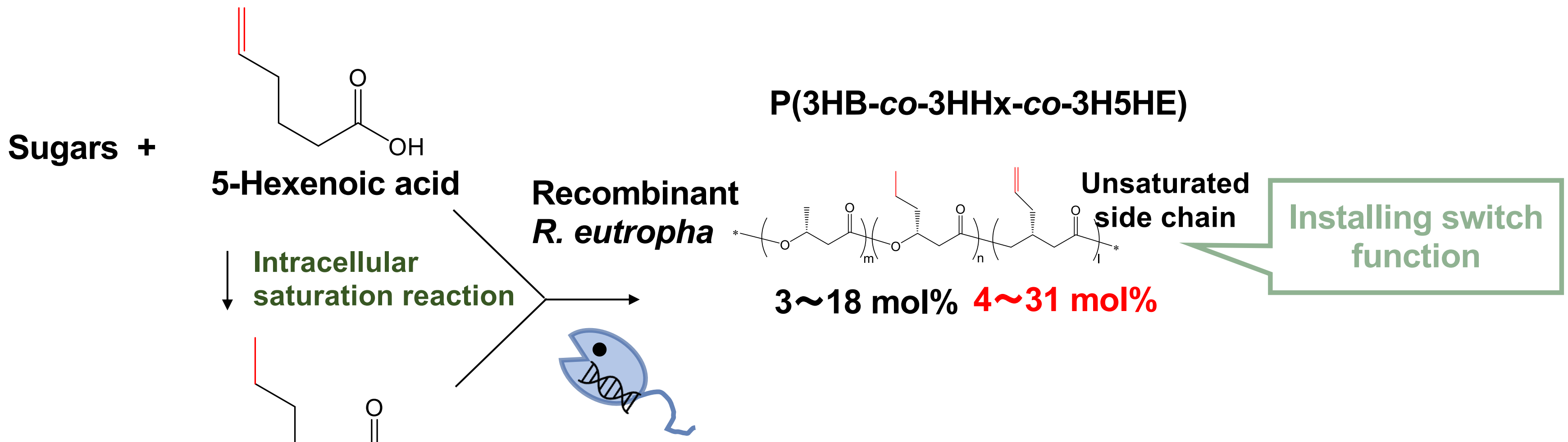
Flask culture（2L）



Extraction

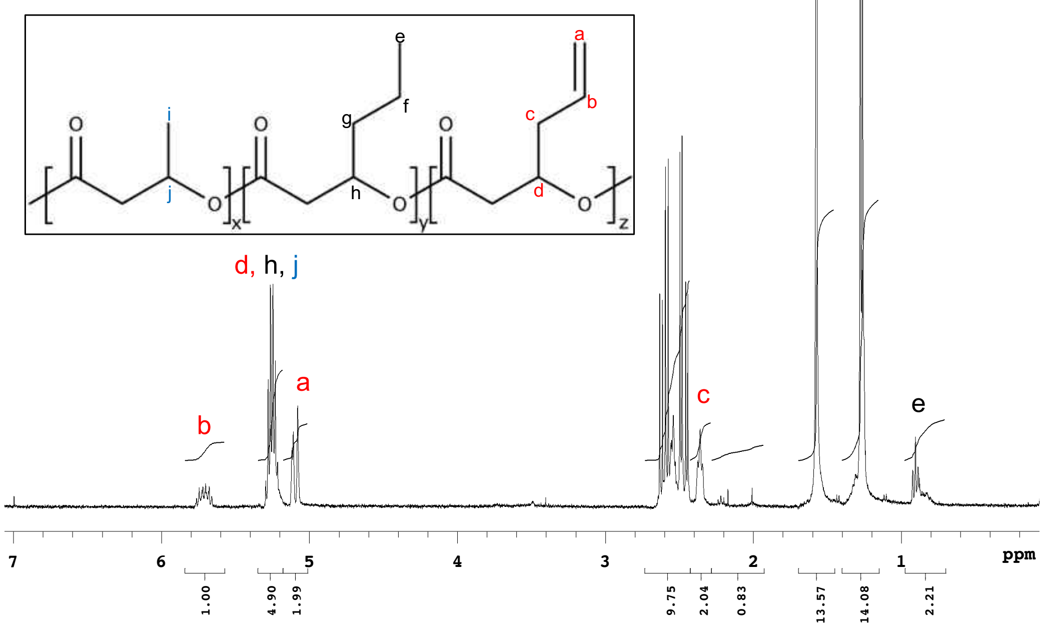
Synthesize 200 g of 3H4PE polymer → switch function can be introduced

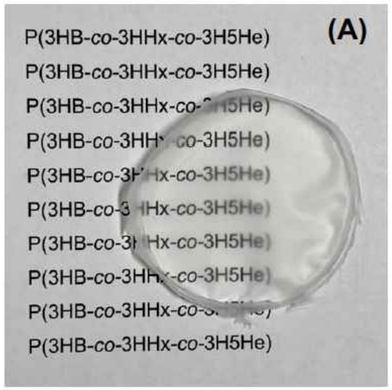
E1 Base materials for installing switching functions



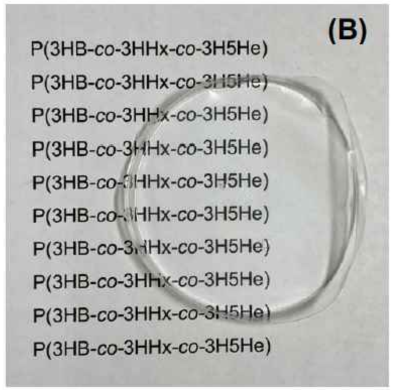
5-hexenoic acid was found to be efficiently converted to 3H5HE in *Ralstonia eutropha* and incorporated into the polymer.

P(3HB-*co*-13mol% 3HHx-*co*-27mol% 3H5HE)



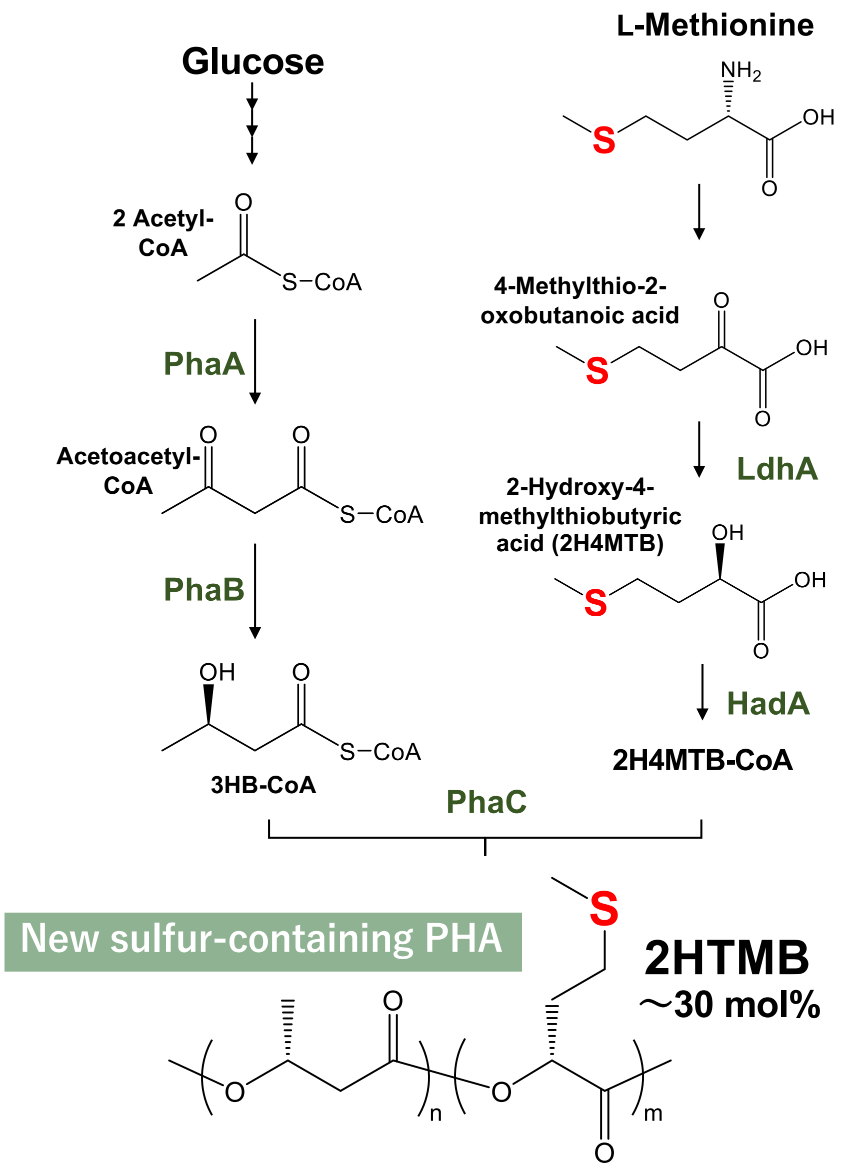


(A) P(3HB-*co*-4.9 mol% 3H5He-*co*-5.2% 3HHx)

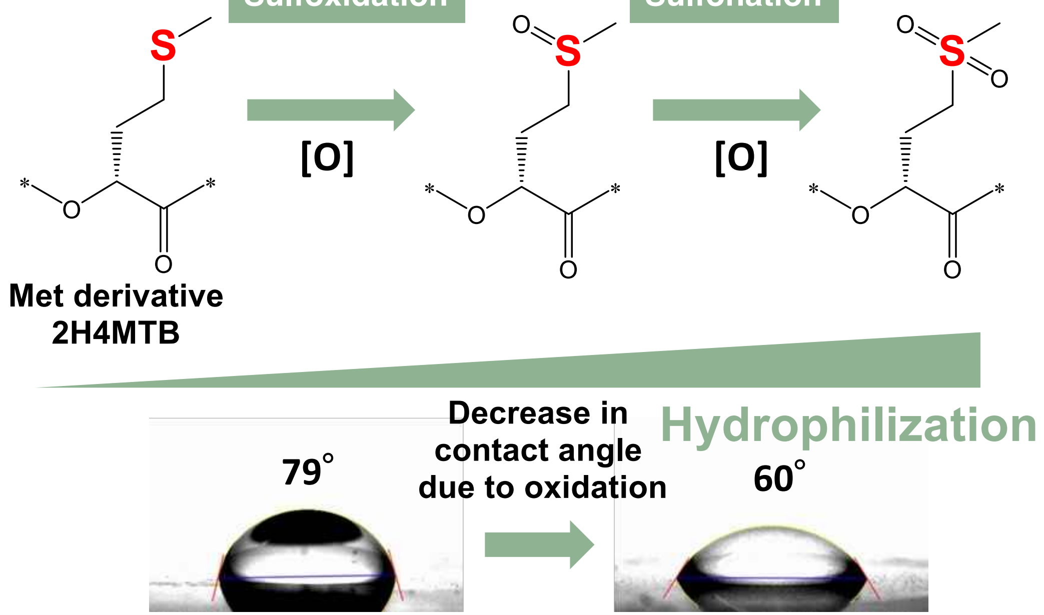


(B) P(3HB-*co*-13.9 mol% 3H5He-*co*-16.3% 3HHx)

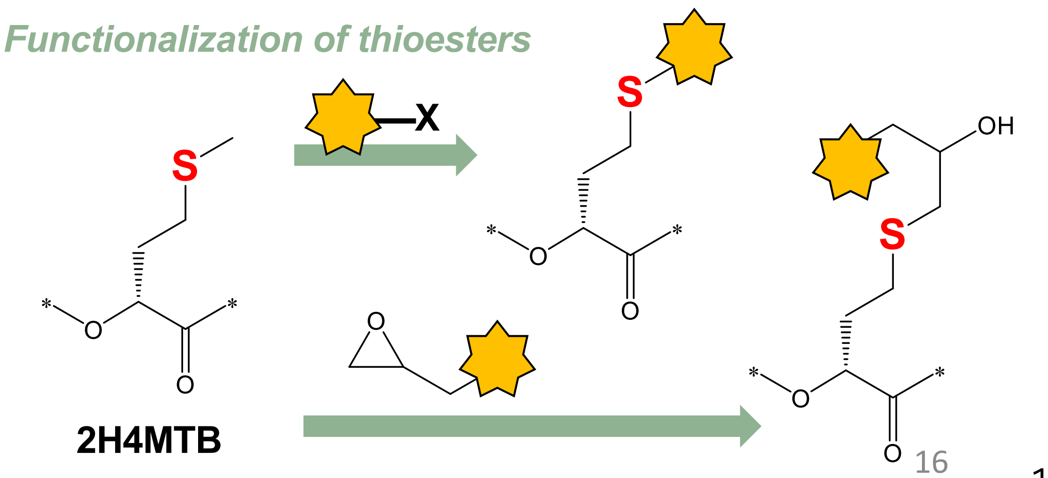
E1 Base materials for installing switching functions



Oxidation

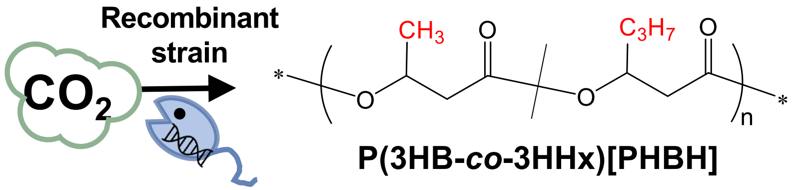


Functionalization of thioesters

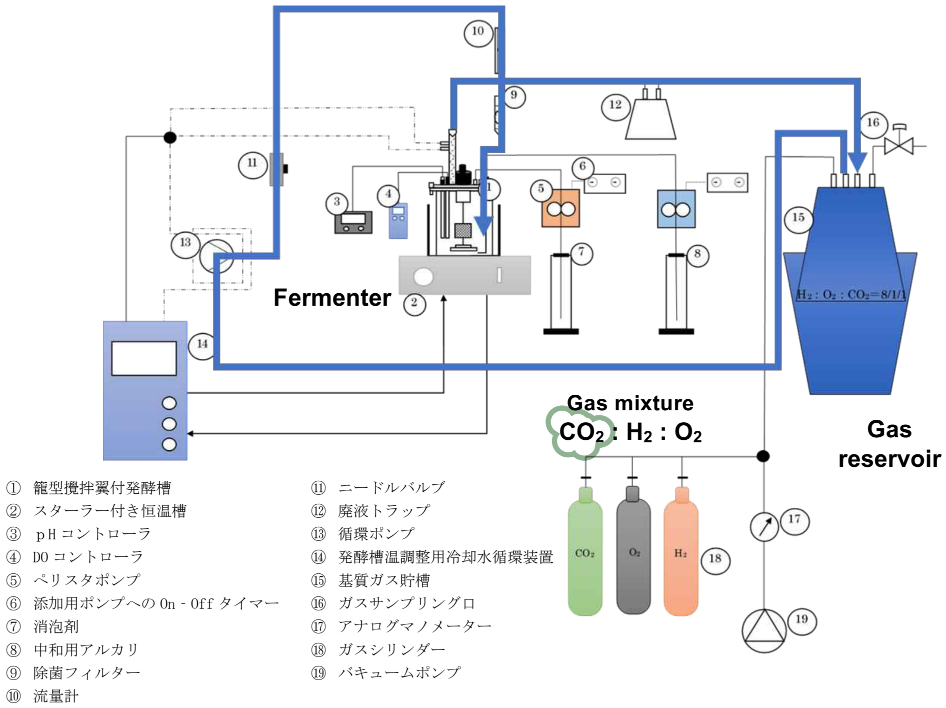


E1 Development of efficient synthesis method for new PHA from CO_2

- Using H_2:O_2:CO_2 gas as a raw material substrate, genetically modified strains of *Ralstonia eutropha* (*C.necator*) were cultured, and a production test of a new polyester with excellent marine degradability is conducted.
- Clarifying the culture characteristics of recombinant strains and developing technology to efficiently produce polyester from CO_2.
- In particular, we will focus on the development of a culture method that enables the improvement of product concentration and speed, and the complete consumption of raw material gas.



P(3HB-*co*-3HHx)[PHBH]



Closed circulation gas culture system

①PHBH productivity of various recombinant strains (flask culture)

Strain / Plasmid	Dry cell wt. (g/l)	PHBH Content (wt%)	Composition (mol%)	
			3HB	3HHx
C.necator H16（wild-type）	17.16	68.2	100.0	0
MF01/ pBPP-ccr$_{Me}$J4a-emd	12.18±0.40	64.0±3.4	94.8±1.1	5.3±1.1
MF01ΔB1/ pBPP-ccr$_{Me}$J4a-emd	10.65±1.35	61.7±4.6	52.3±6.2	47.7±6.2
MF01/ pBPP-ccr$_{Me}$JAc-emd*	11.22±2.67	64.6±8.1	88.7±6.4	11.3±6.4
MF01ΔB1/ pBPP-ccr$_{Me}$JAc-emd	8.52±1.00	67.8±1.8	87.1±2.3	11.1±1.3

* 3HHx 10mol% PHBH is known to have the best physical properties （~ 2020）

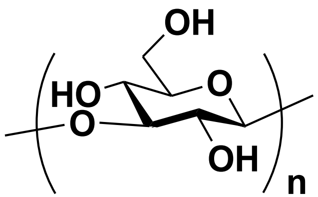
② PHBH production by *C. necator* strain MF01/Jac (Jar fermenter)

Dry cell (g/L)	PHBH (g/L)	3HB (mol%)	3HHx (mol%)	Culture time (h)	Productivity (g/L/h)
61.4	51.5	94.6	5.4	205	0.300
71.0	58.4	86.2	13.8	119	0.594

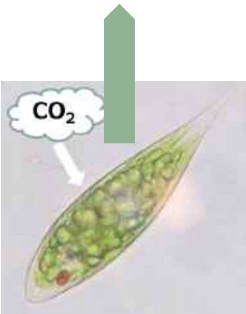
- Search for mineral salt medium composition suitable for PHBH biosynthesis and control of inorganic nutrient concentration during culture
- Productivity improvement by improving fermentation tank agitation performance, etc.

（*Highest value as of November 2022*）

E1 Marine biodegradable plastics produced from polysaccharides



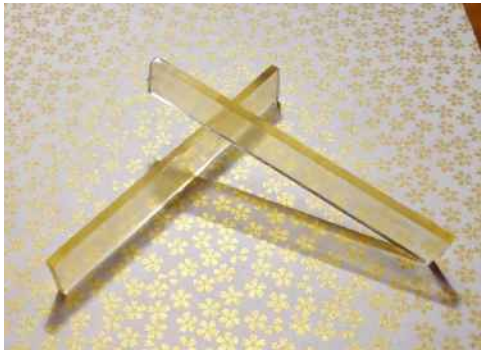
Paramylon



Euglena

Extraction
Thermo-processing

- **Esterification**
- **New processing procedure**



Injection molding

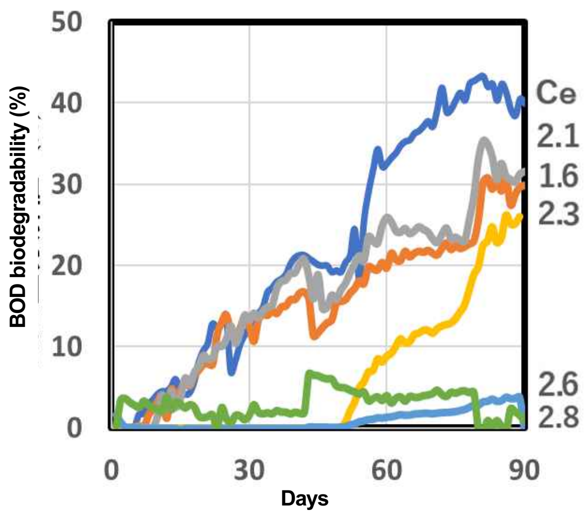
- Resistant to acids and alkalis
- Better impact strength > PP

Melt-spun fibers

- Processable without additives
- High-strength

BOD biodegradation test

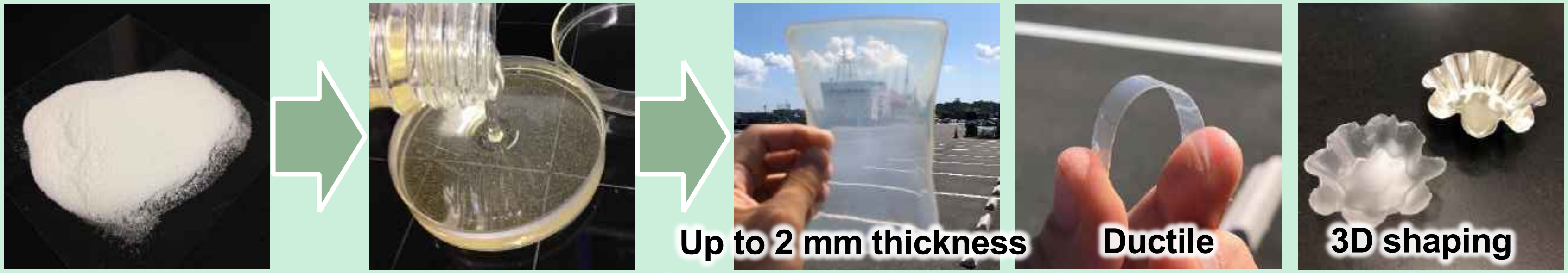
Using Seawater from Tokyo Bay
Successful development of new high-performance materials with controlled marine degradability from polysaccharides



Good biodegradability

Non-biodegradability

E1 Development of new cellulose-based transparent materials

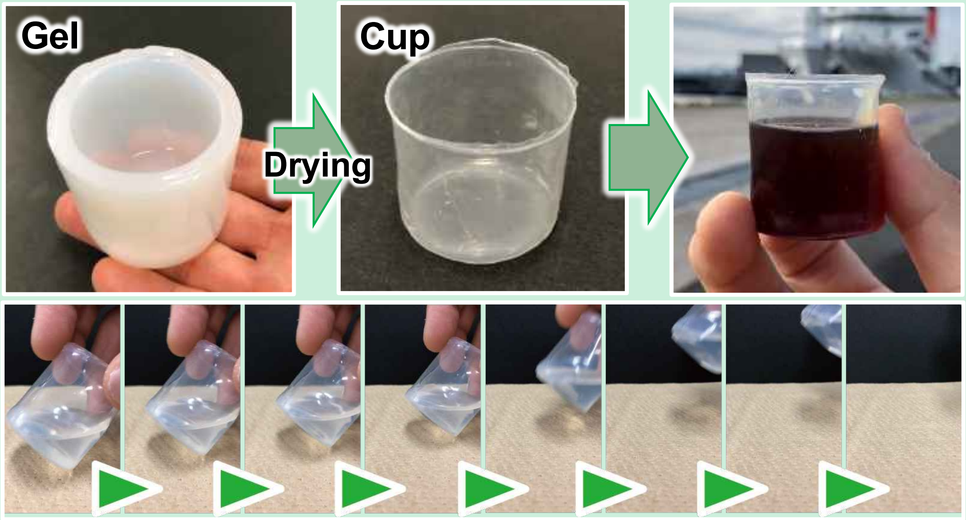


Dissolving, coagulating, and drying cellulose gives transparent paperboard.

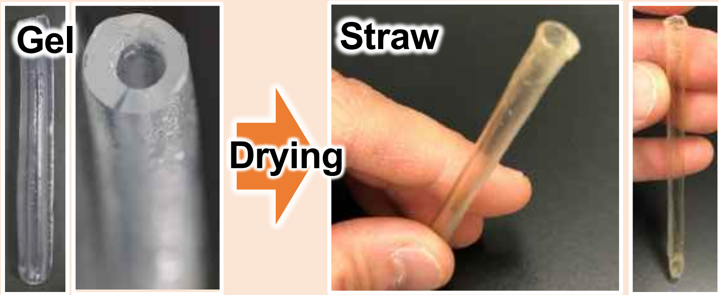
Compositionally identical with paper but more functional.

PCT/JP2020/03984

A transparent cup made of cellulose

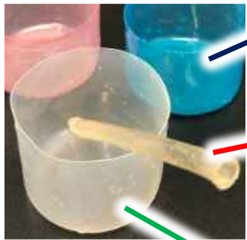


The cup holds water without inner film, which is necessary for the conventional paper cup.



The fragileness of chitin was overcome by the improved molding process.

By the improved shaping process, the preparation of materials entirely made of pristine cellulose or chitin was successful.



Easy-coloring

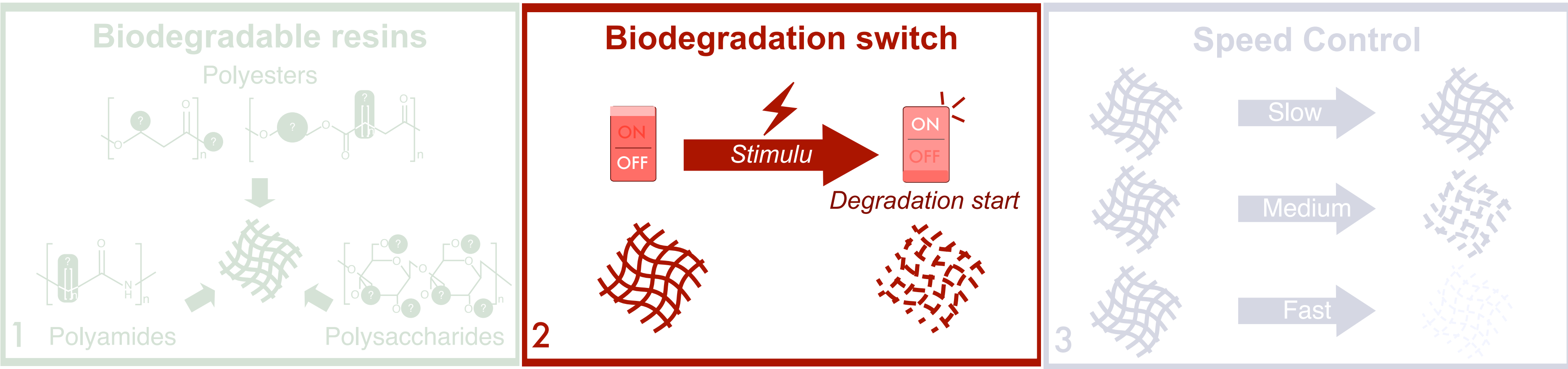
Chitin straw: Complete decomposition in 2 months.

Cellulose cup: Complete decomposition in 10 months.

E2

8. Major results regarding ongoing topics

E2, Development of switching function to start biodegradation

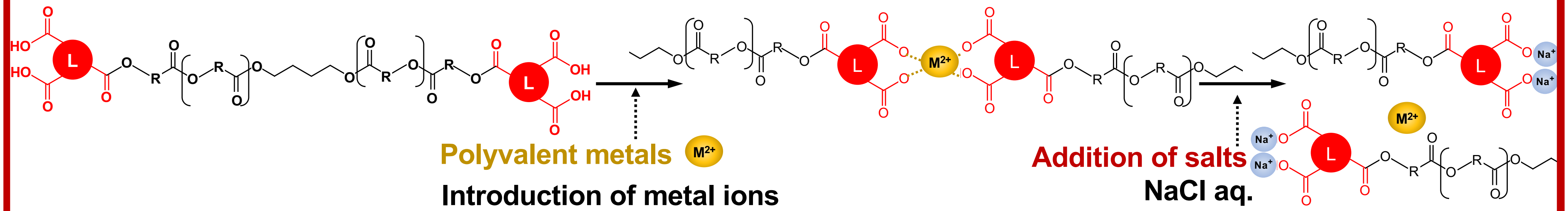


E2：	**Development of switching function**	**pH** **Salt conc.** **ORP** **Pressure** **Temperature** **Wearing**

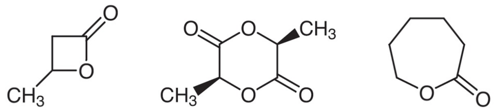
E2 Switching triggered by difference in salt conc.

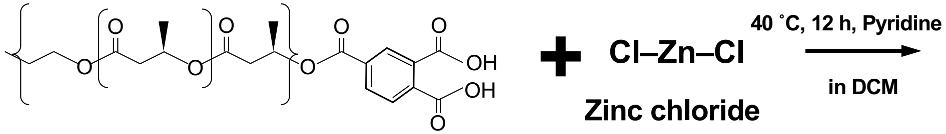
Polyesters introduced the ligands with dicarboxylic acid group

Formation of coordinate bonds Cleavage of coordinate bonds

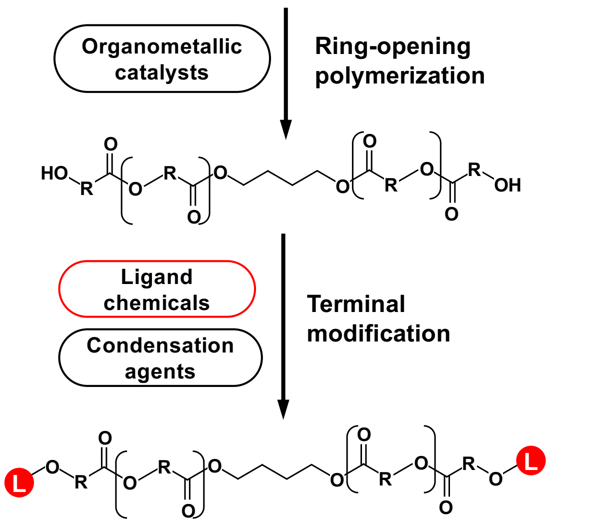


Lactones and Lactide monomers





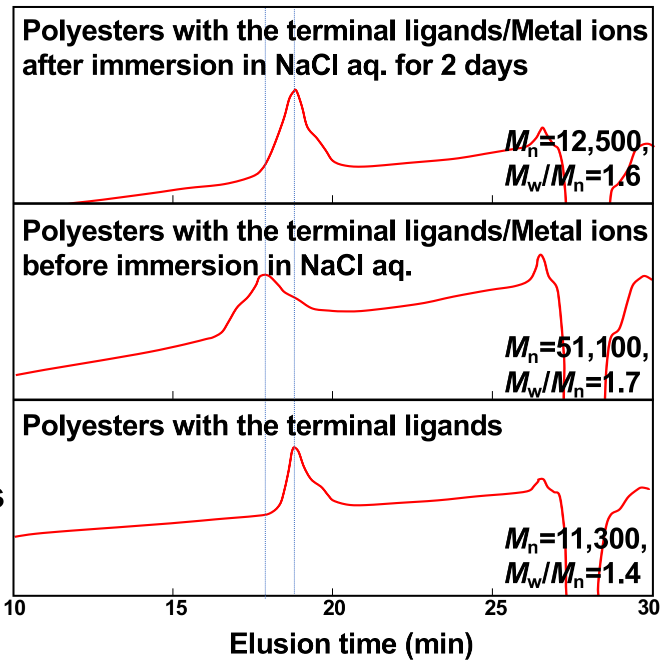
Linear polymers forming coordinate bonding between terminal ligands and metal ions

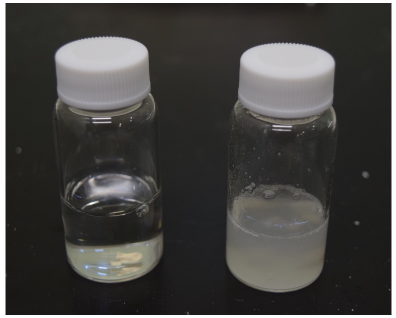


Polyesters with the terminal ligands

Succeeded in introduction of ligands into the chain-ends of aliphatic polyesters

Changes in molecular weights determined by GPC

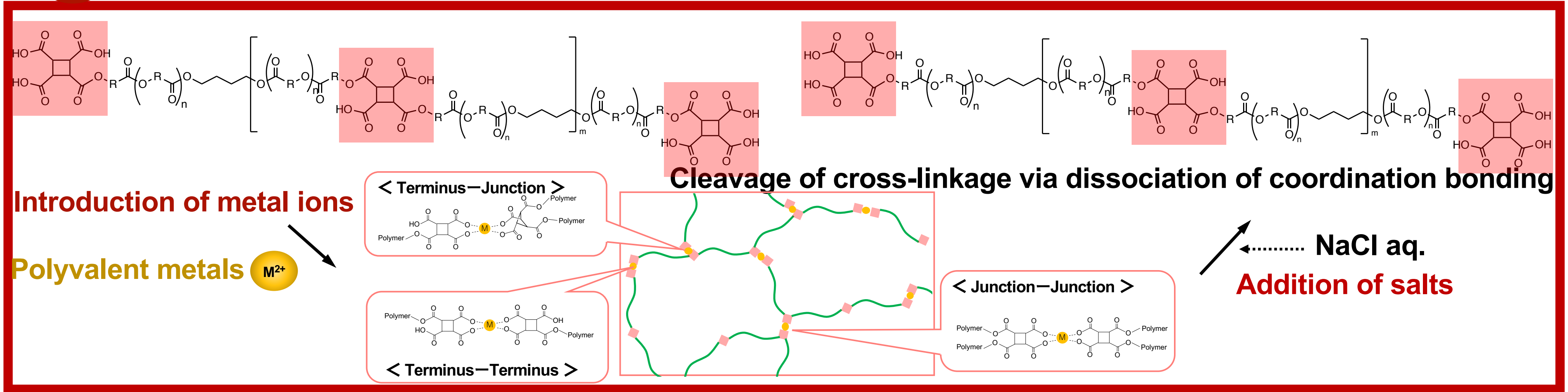


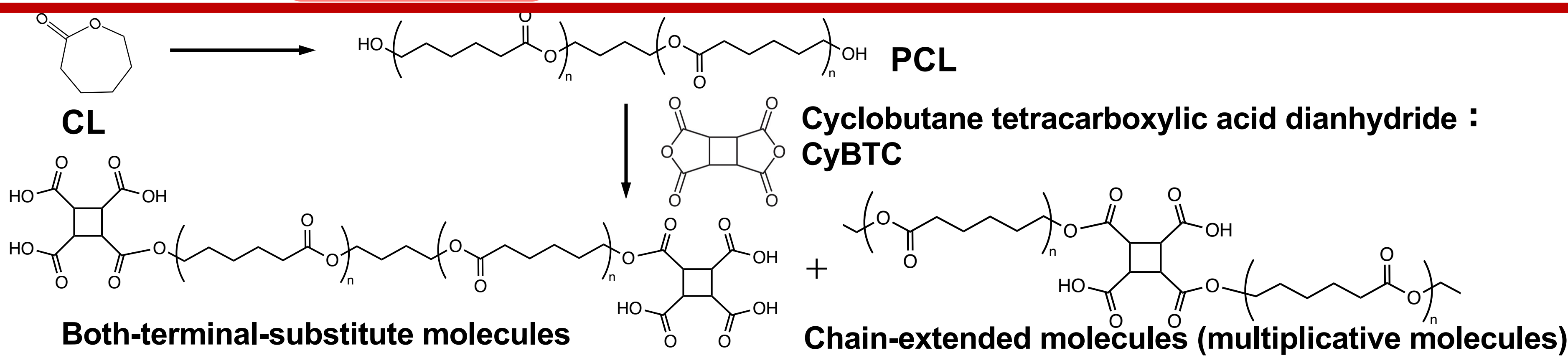


Water 3wt% NaCl aq.

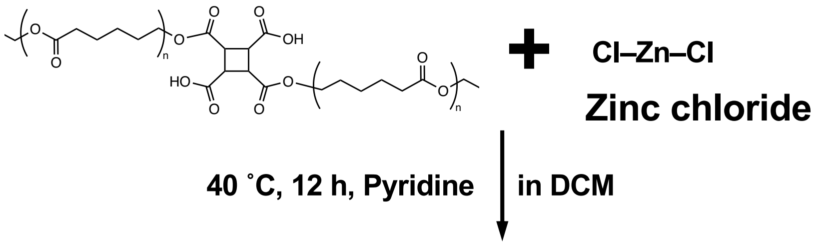
Confirmed the cleavage of coordinate bonding of polymers by the immersion into NaCl aq. solution with a concentration of 2wt% or more.

E2 Switching triggered by difference in salt conc.



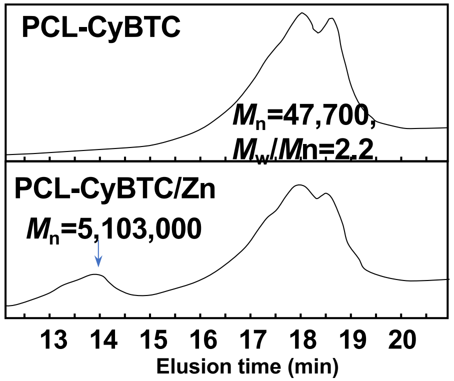


PCL with cyclobutane tetracarboxylic acid



Cross-linking polymers forming coordinate bonding between ligands and metal ions

<u>Changes in molecular weights determined by GPC</u>

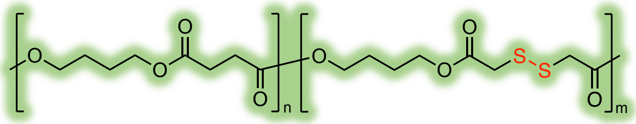


Confirmed the partially cross-linking structure via formation of coordinate bonding between metal ions with tetrahedral coordination and the functional ligands of dicarboxylic acid group in polyesters

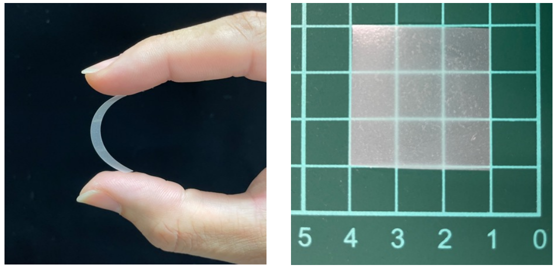
Reaching the seafloor sediment and degradation switch is turned on

E2 Switching triggered by difference in ORP

Biodegradability control by low oxidation-reduction potential (ORP) in marine environments

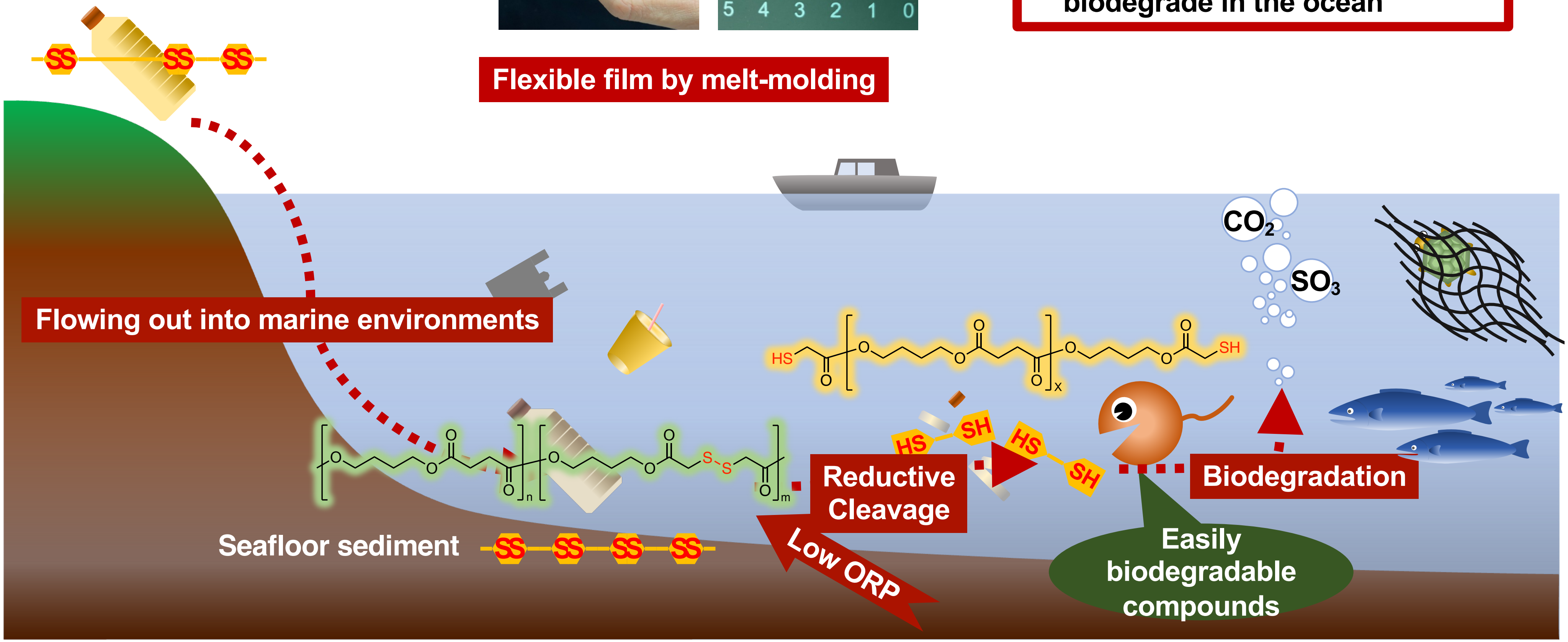


PBSDT

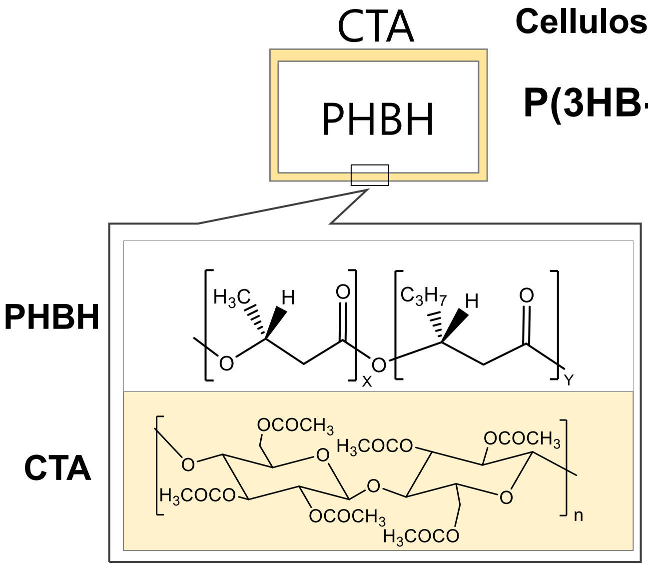


Flexible film by melt-molding

- PBSDT as an analogue of PBSA
- Reductive cleavage of disulfide bonding in low ORP condition
- Reduced degradation products biodegrade in the ocean



E2 Switching triggered by difference in pH



Cellulose triacetate (CTA)coating

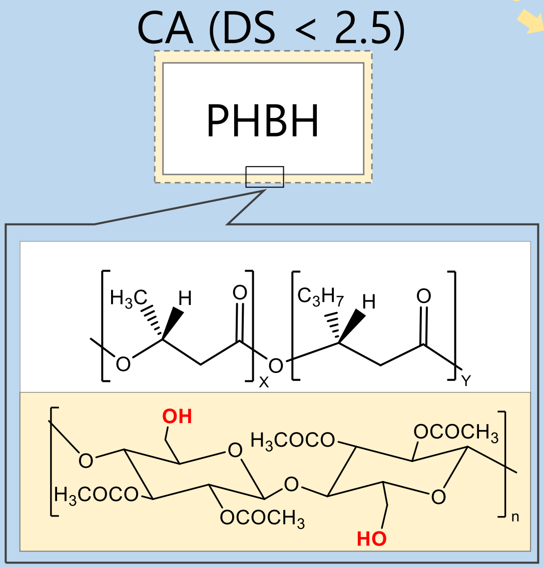
P(3HB-*co*-3HHx)

No degradation for high DS CA (CTA)

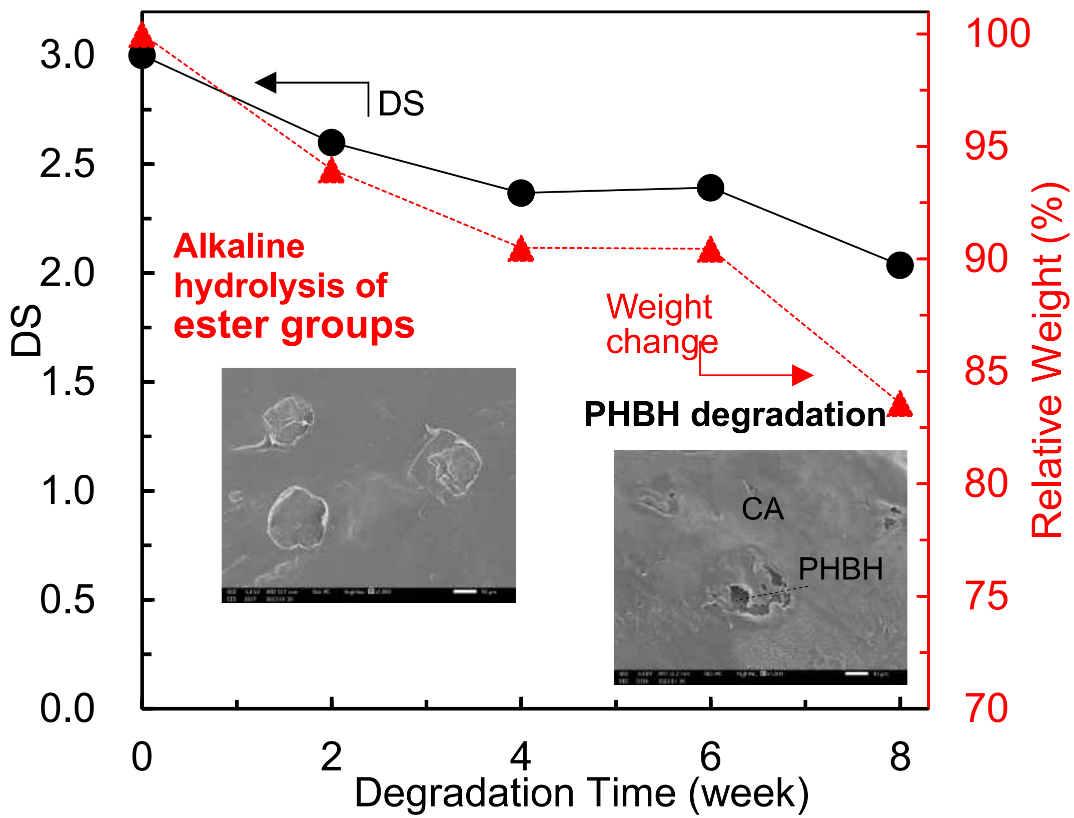
Switch ON

Alkaline hydrolysis in seawater with pH 7.5-8.3

CH_3COOH



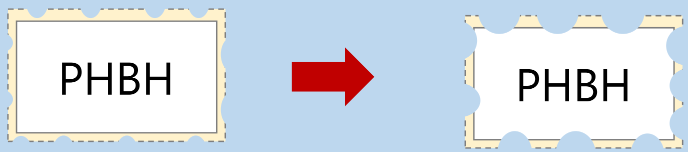
Degradation test in sea water



CA biodegradation after alkaline hydrolysis of acetyl groups and subsequent decrease in degree of substitution (DS)

After 4 weeks

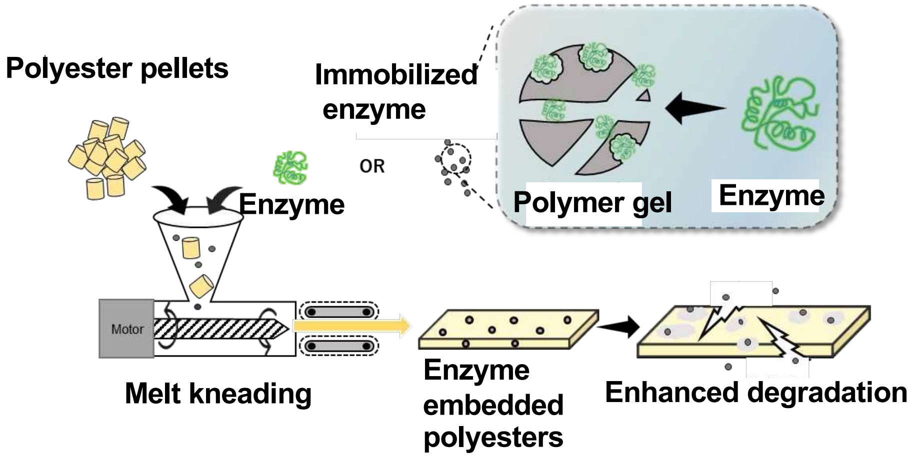
After 8 weeks



CA biodegradation

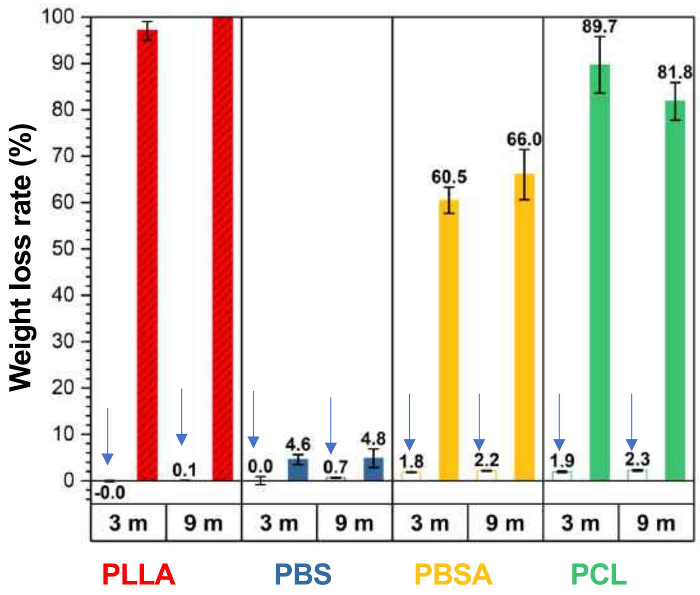
PHBH degradation triggered by CA degradation

E2 Switching triggered by wear (Enzyme)



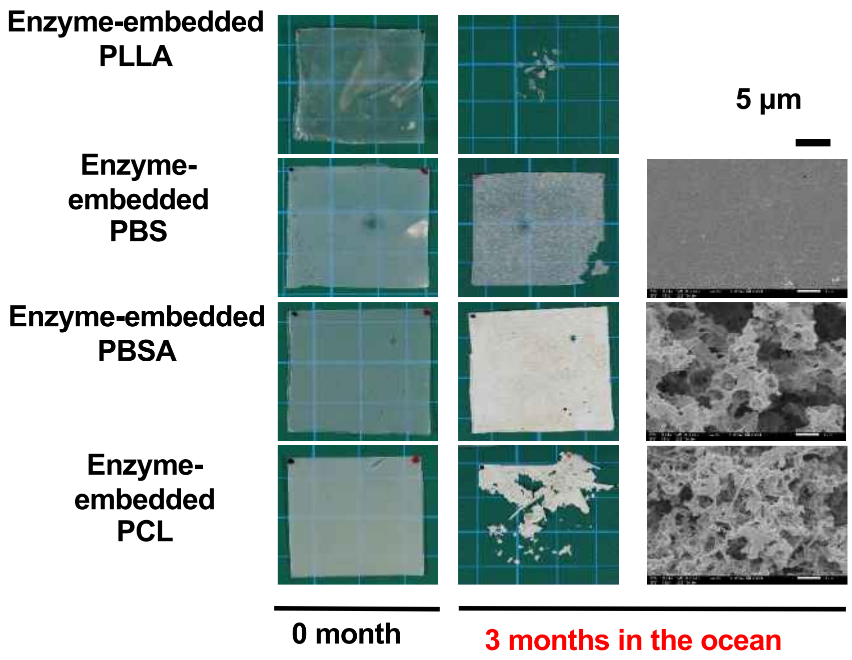
Enzymes (Lipase, Cutinase, proteinase K) were embedded in biodegradable plastics that show slow degradation in the ocean.

Enzymes were immobilized in gel-beads to improve stability against heat molding.

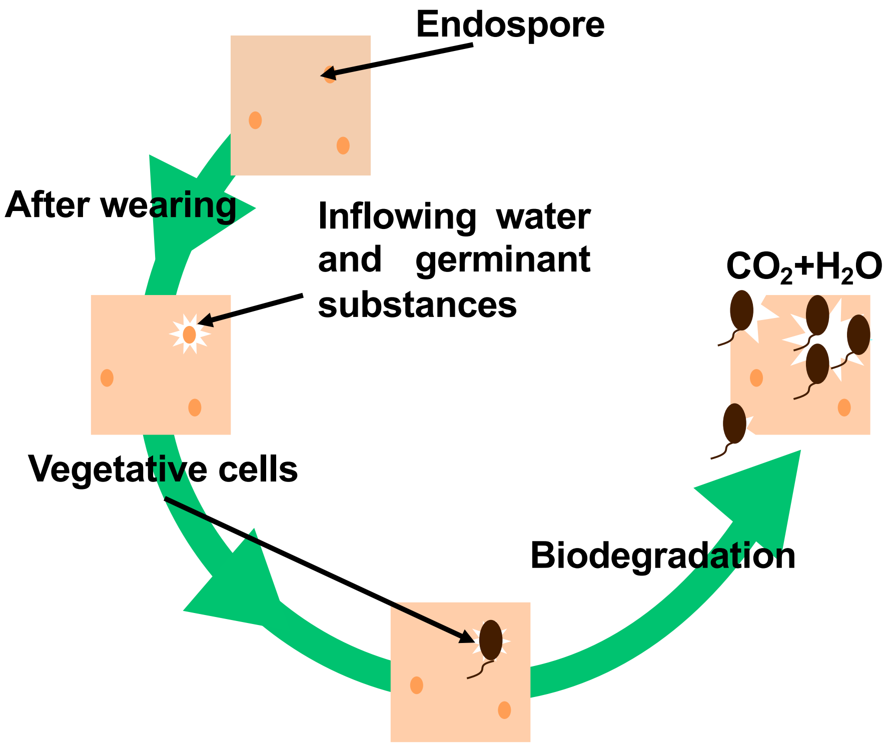


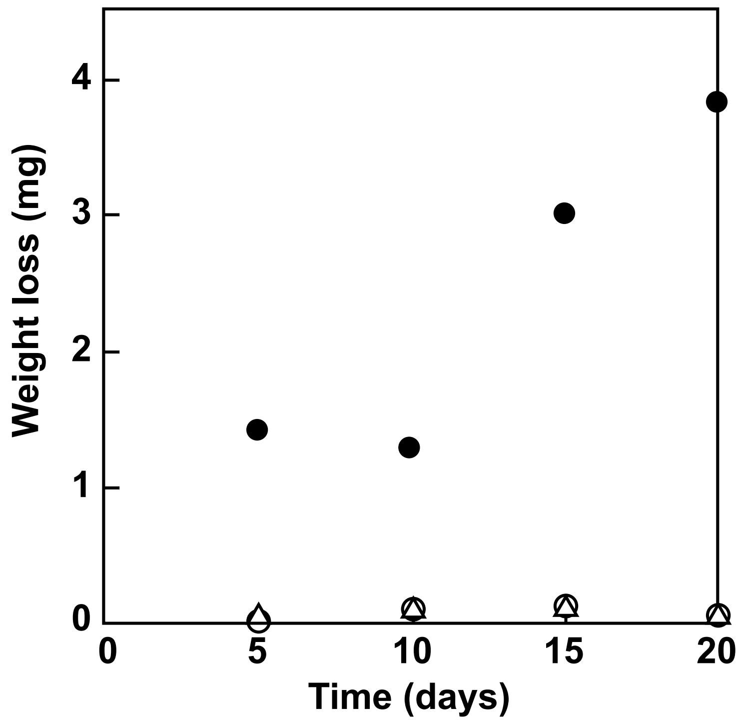
Without enzyme: no degradation

Enzyme-embedded polyesters: High weight loss (%) and degradation



E2 Switching triggered by wear (Endospore)





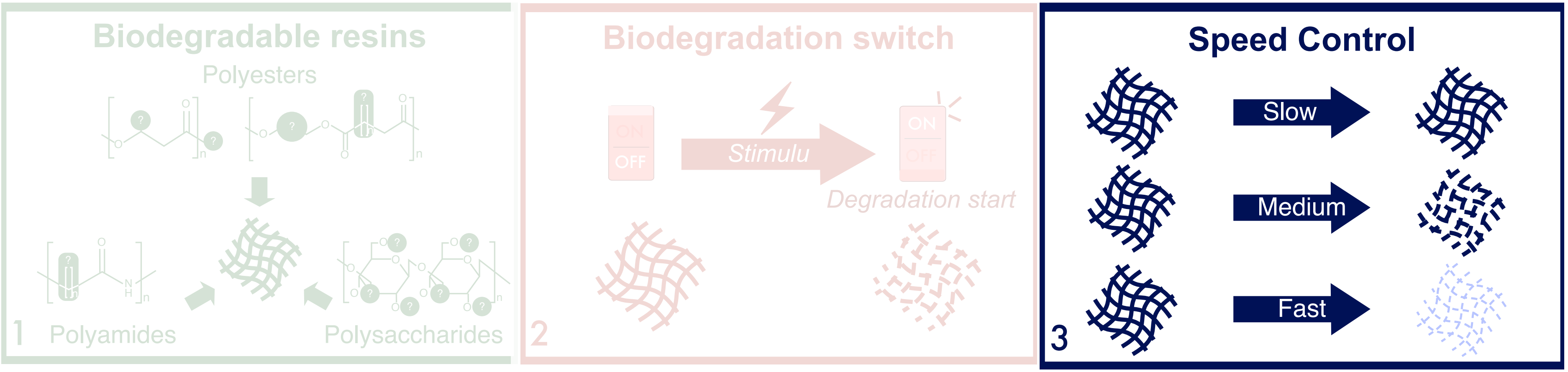
Degradation test of spore-containing PESu ●: Weight loss of spore-containing PESu film with Yeast extract (YE). ○: Weight loss of PESu film with YE. △: Weight loss of spore-containing PESu film without YE.

- **Degradation is triggered by wear, and biodegradation proceeds as endospores transform to vegetative cells.**

E3

8. Major results regarding ongoing topics

E3, Biodegradation rate control

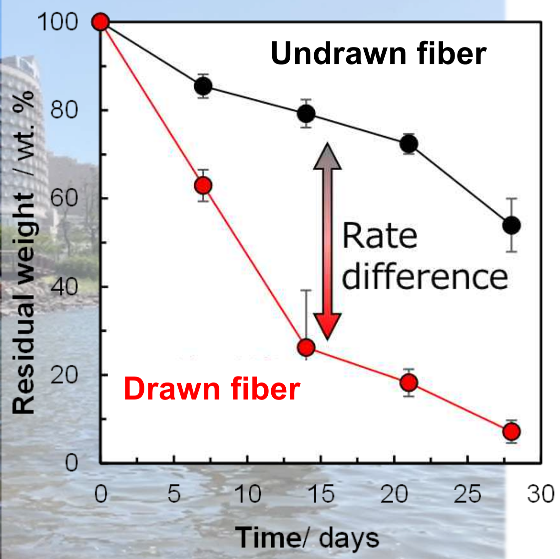


E3：	Biodegradation rate control	Acceleration	Biological factor
			Material science
		Deceleration	Material science

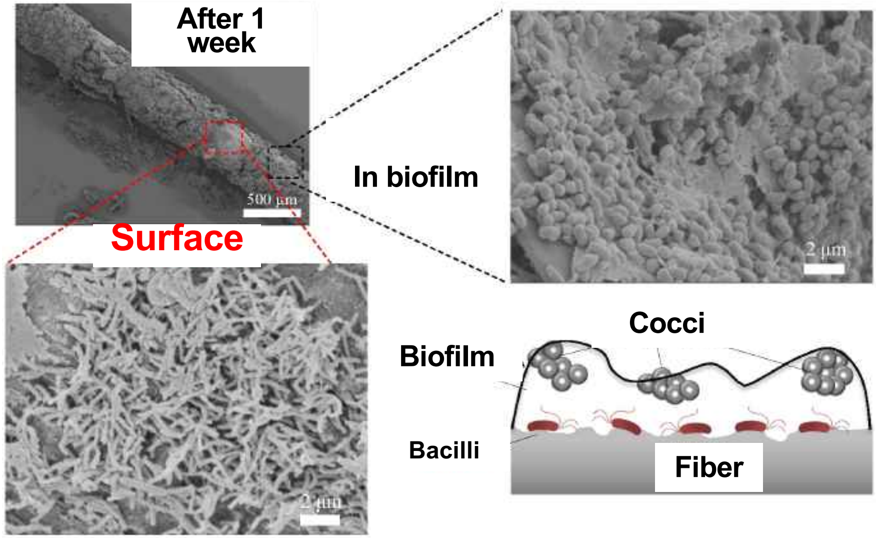
E3 Biodegradation rate factors from materials science

High-strength and modulus PHA fiber

Microbial polyester fibers	Mechanical properties: Tensile strength /MPa	Young's modulus / GPa	Elongation at break / %
P(3HB)	1320	18.1	35
P(3HB-*co*-8 mol%-3HV)	1065	8.0	40
P(3HB-*co*-9 mol%-3HH)	552	3.8	48



Biodegradation of fiber

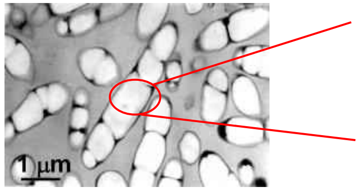


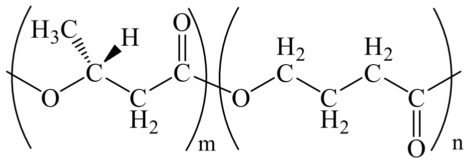
Bacilli on the fiber surface may degrade fiber using the enzyme.

- Degradation rate can be controlled by drawing ratio.
- Degradation rate is related to crystalline morphology.

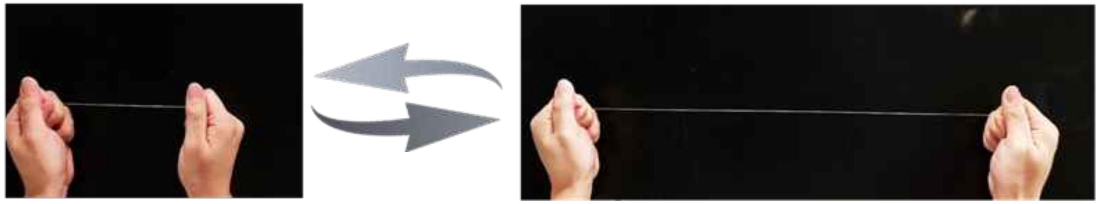
E3 Biodegradation rate factors from materials science

Highly stretchable biodegradable fibers

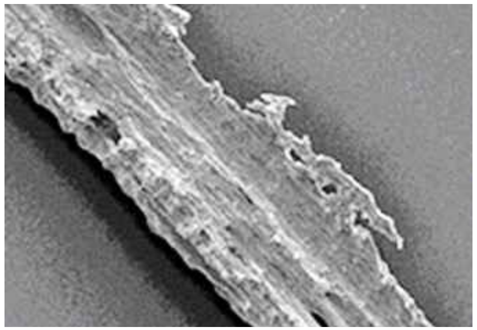




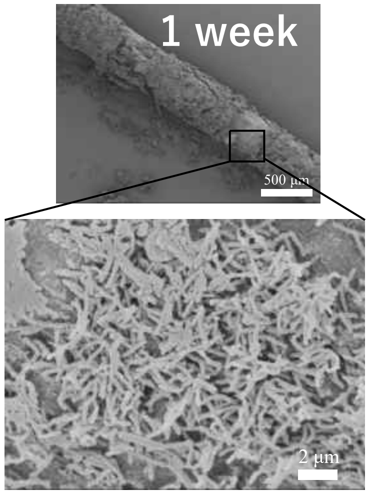
P(3HB-*co*-4HB)



Surface observation

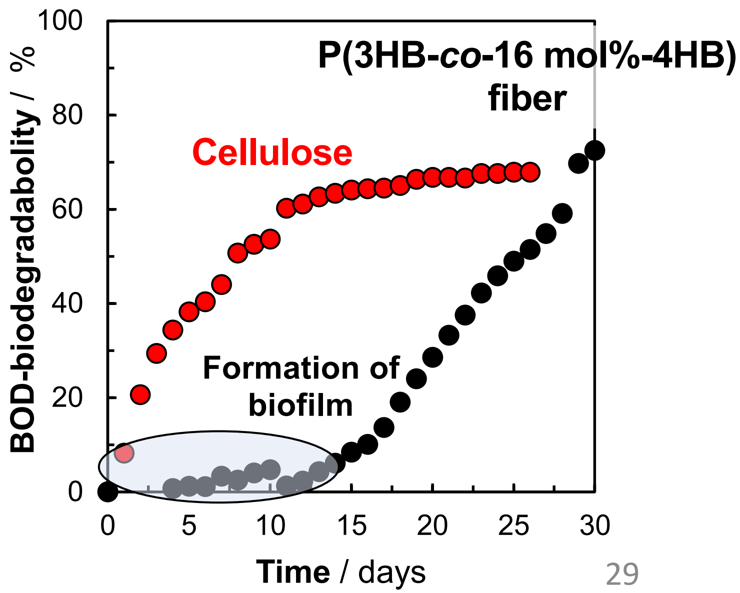


SEM image of the fiber during degradation

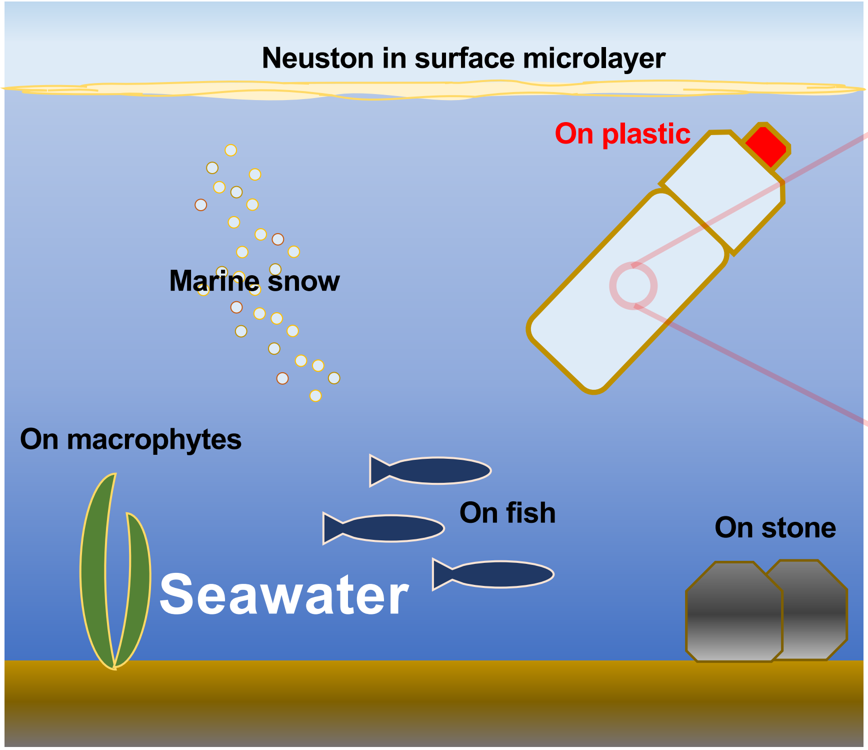


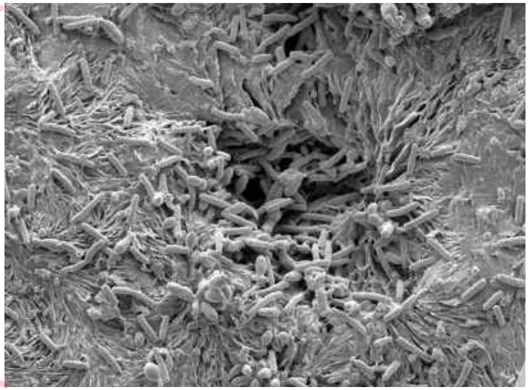
Microorganisms on fiber surface

BOD biodegradability in sea water



E3 Plastisphere：Microbial flora formed on plastic surface

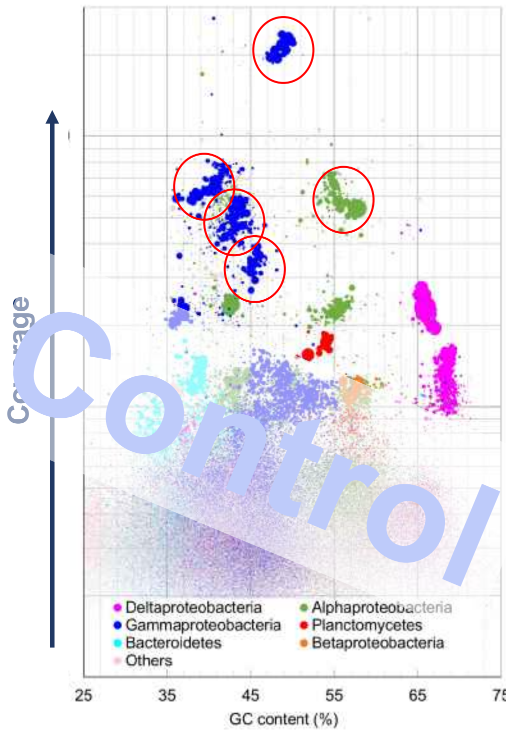




SEM image of plastisphere formed on the biodegradable plastic surface.

Metagenome analysis

Microbial accumulation in the plastisphere



Microbes with high abundance
= Genome information of **microbes involved in the biodegradation**.

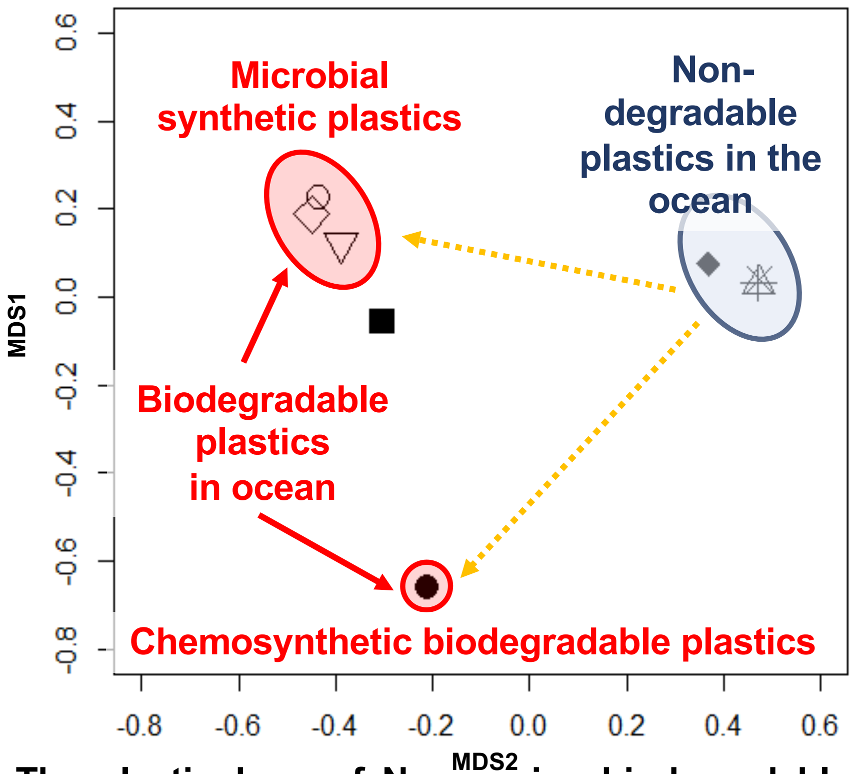
Elucidate the biodegradation mechanism of plastic and control the degradation rate.

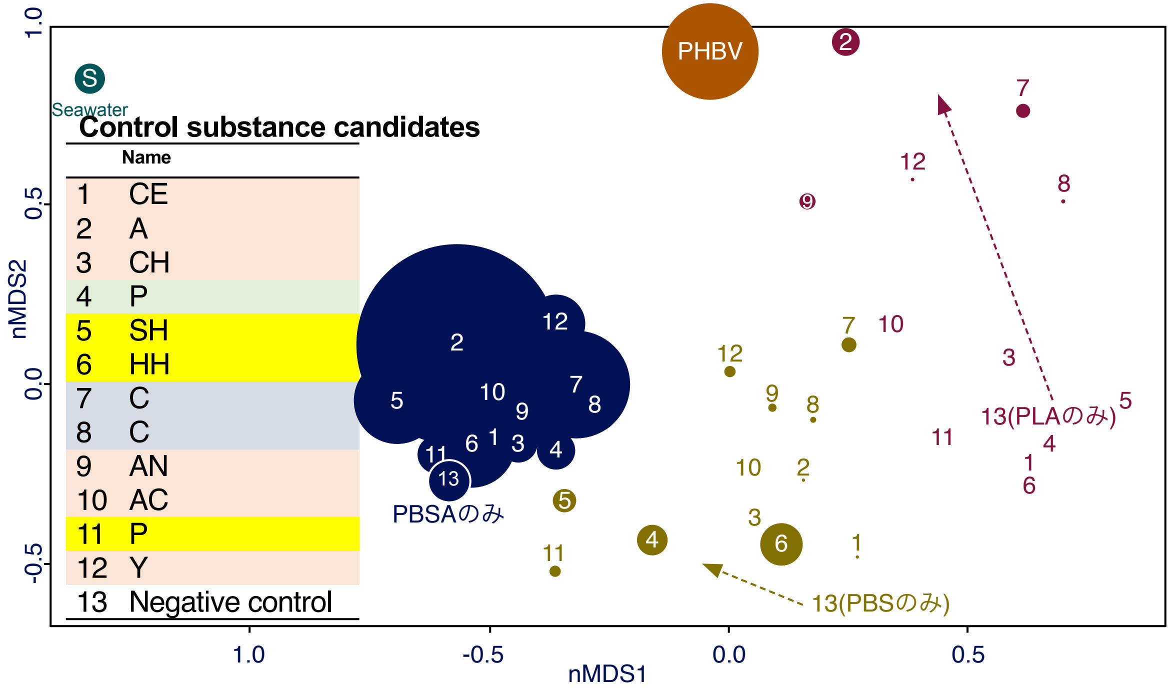
Also investigate the plastisphere in non-oceanic environments.

Biodegradable plastics is exposed to pond.

E3 Biodegradation rate control by controlling plastisphere

Addition of 10% plastisphere control substance candidate to the biodegradable base polymer.
The films were exposed to seawater and investigated weight loss and change in plastisphere.





The plastisphere of Non-marine biodegradable plastics close to that of marine biodegradable plastics.→ Improving biodegradability

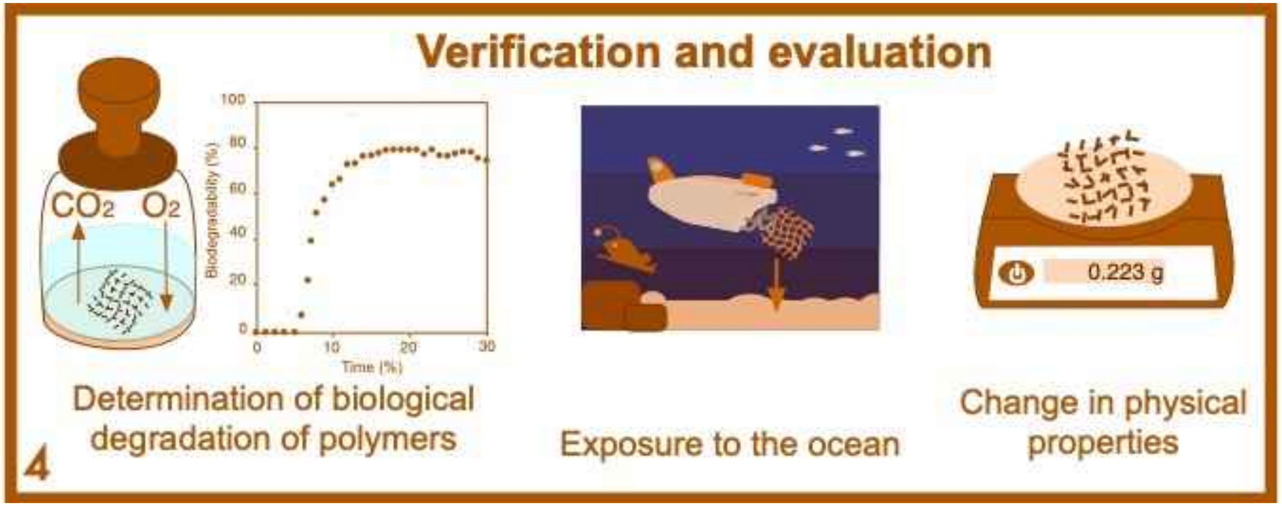
Non-metric multidimensional scaling (nMDS) based on the Bray-Curtis index. Numbers in the plot indicate the type of substance. The area of the plot shows the biodegradation rate except for seawater.

PBSA
No.2、No.5、No.6、No.7
PBS
No.5、No.6
PLA
No.2、No.7、No.9
Effect on increase in the degradation rate.

E4

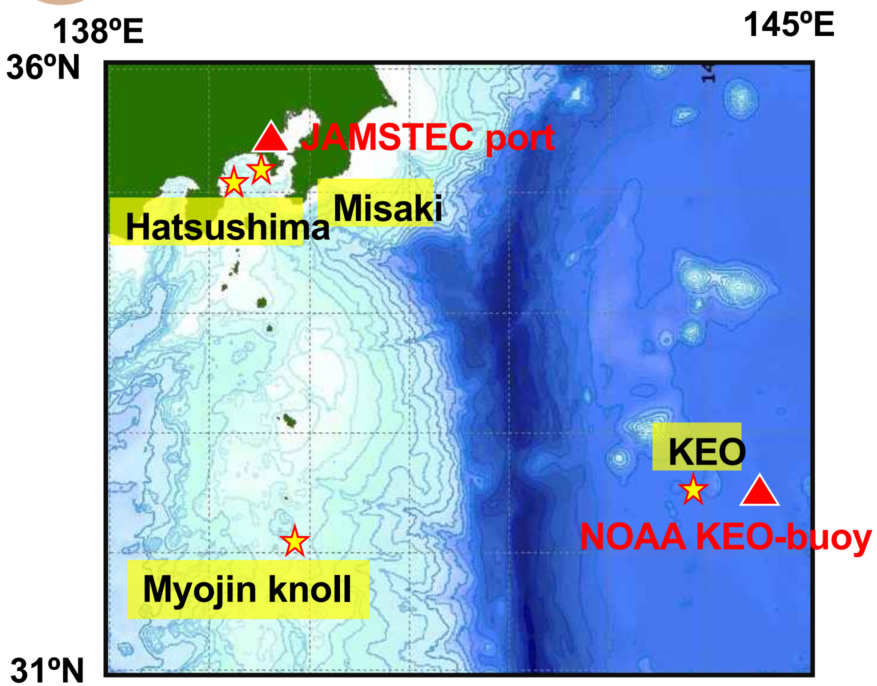
8. Major results regarding ongoing topics

E4, Validation and evaluation of biodegradability in laboratory and deep-sea environments



E4： **Verification and evaluation of marine biodegradability**	**In vitro**	BOD biodegradability Test condition
	In vivo	In Shallow water In deep-sea Buoy

E4 *In situ* biodegradation tests of novel materials



■ We carried out 6 cruises to test the biodegradability of newly developed materials on the deep-sea floor from 2020 to 2022. This is the only project that is testing biodegradability on the deep-sea floor in situ, where large amount of plastic debris are accumulating.

■ We have also started biodegradability test at the surface of the North Pacific pelagic site.

■ The recovered materials were examined with different chemical and physical tests, together with meta-omics approaches of the attached biofilms.

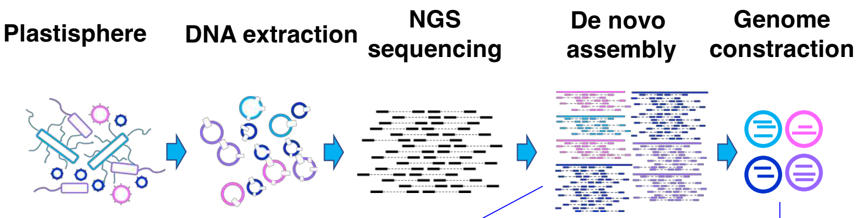
Deployment and recovery with the manned submersible

Plastic chambers deployed on the deep-sea floor

Plastic chambers deployed on the mooring buoy

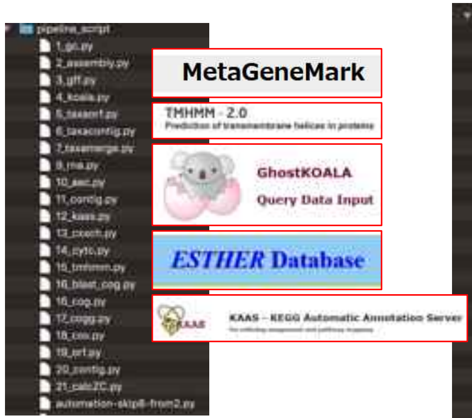
E4 Meta-omics analysis of plastisphere correlating with biodegradation

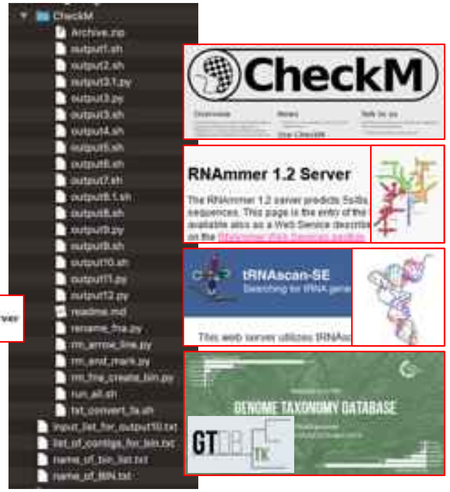
Pipeline



Pipeline for genome quality control

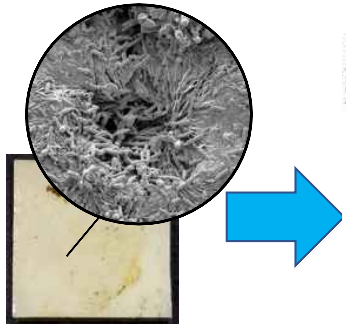
Pipeline for analysis the genome and gene function

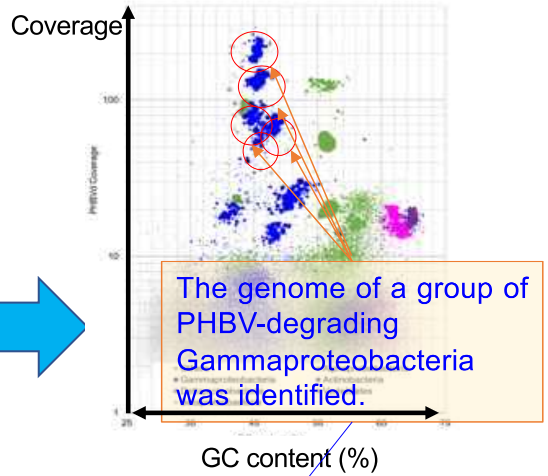




Metagenome analysis of plastisphere

Marine PHBV plastisphere





Function	ESTHER ID	Gamm1	Gamm2	Gamm3	Gamm4	Gamm6	Gamm7	Gamm8	Gamm5	Gamm9	Gamm10	Alph1	Alph2	Alph3	Alph4	Alph6	Acti1	Delt1	Alph5
Esterase_phb	Esterase_phb	23	13	19	20	18	14	1	0	3	1	0	0	0	0	0	1	6	0
Abhydrolase_6	PHB_depolymerase_PhaZ	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0
Esterase_phb_PHAZ	Esterase_phb_PHAZ	1	0	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0
Thioesterase	Thioesterase	9	9	4	4	8	8	13	5	0	0	1	0	0	2	2	6	10	0
Lipase_2	Lipase_2	10	2	6	10	11	5	0	1	0	0	0	0	0	0	0	0	1	0
Abhydrolase_6	6_AlphaBeta_hydrolase	15	12	12	18	19	14	14	32	11	15	9	6	16	32	24	50	31	0
Bacterial_lipase	Bacterial_lip_Faml.3	4	0	1	3	2	1	2	2	2	1	5	5	10	0	25	1	0	3
AlphaBeta_hydrolase	AlphaBeta_hydrolase	2	5	2	2	4	4	1	3	3	0	6	3	5	3	4	9	8	0
Hormone-sensitive_lipase_	Hormone-sensitive_lipase_like	1	0	1	2	1	4	0	1	8	1	2	0	1	9	7	12	6	0
Epoxide-hydrolase_like	Epoxide_hydrolase	2	2	1	2	1	1	1	0	5	0	0	0	1	3	3	10	5	0
Peptidase_S9	Prolyl_oligopeptidase_S9	3	1	3	4	3	0	2	10	0	1	0	0	0	4	0	0	3	2
Hydrolase_4	Proline_iminopeptidase	3	1	1	3	2	2	1	4	1	1	1	1	1	1	1	3	8	0
Dienelactone_hydrolase	Dienelactone_hydrolase	1	1	0	1	2	1	1	2	1	1	0	0	1	2	1	8	3	0

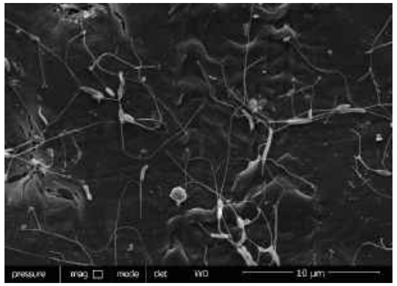
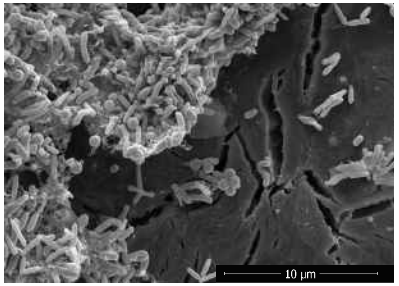
The genome code many PHB depolymerase genes

We could construct a method to analyze a large amount of data efficiently at high speed.

We could identify the candidate of PHBV-degrading Gammaproteobacteria and their PHBV depolymerase genes using meta-omics analysis.

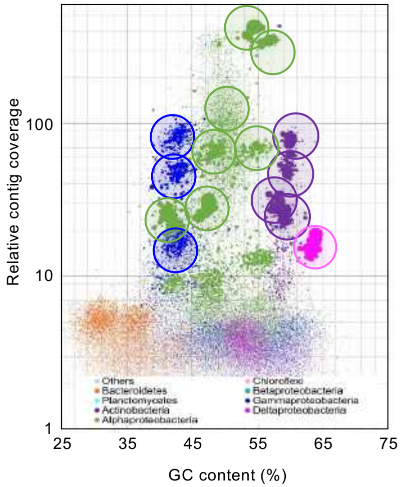
E4 Meta-omics analysis of plastisphere correlating with biodegradation

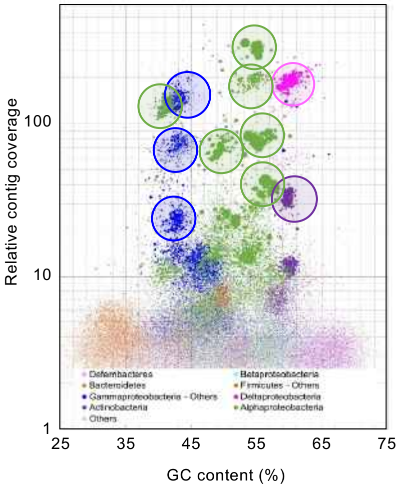


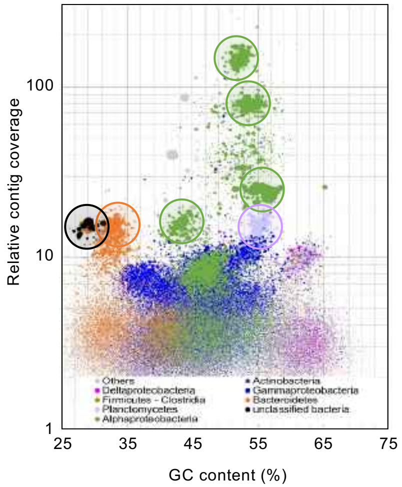



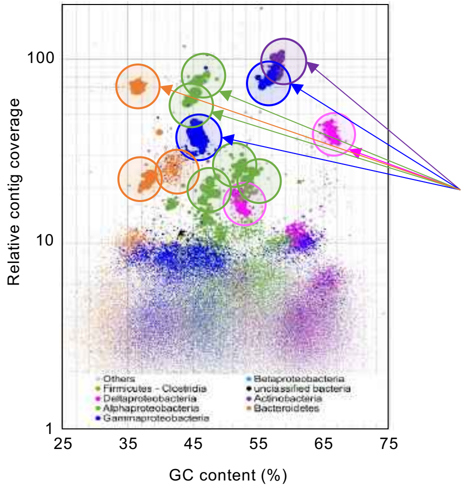
Misaki (BMS) | Hatsushima (BHT) | KEO (AKR) | Myojin knoll(BMJ)

High
Relative frequency
Low



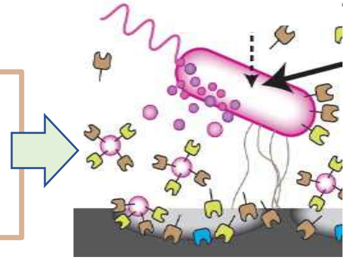




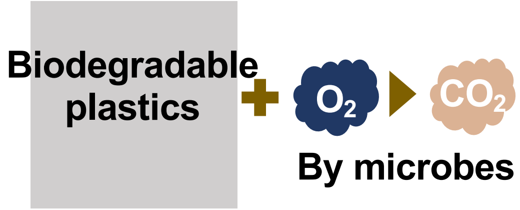


Genome of dominant microbes (Metagenome-assembled genomes, MAGs)

- ✓ Different and diverse plastisphere microbiomes formed on a marine biodegradable plastic at different sea area.
- ✓ Genomes (MAGs) of dominant microbes were recovered from the plastisphere metagenomes.
  - ➔ **Growth of microbes related to biodegradation of plastic**
  - ➔ **Toward identification of enzymes for marine biodegradation**



E4 *In vitro* biodegradation tests of novel materials

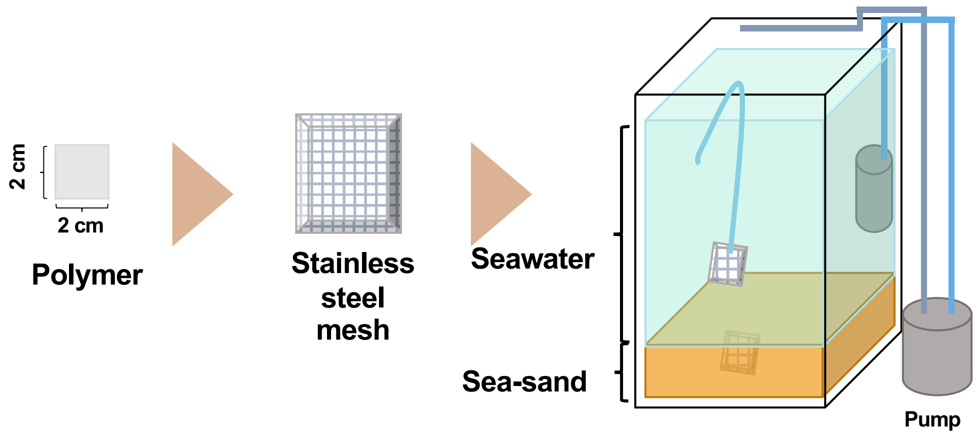


$$Biodegradability\% = \frac{O_2}{Th\,OD}\times 100$$

O_2: Biological oxygen demand (BOD) used for catabolism of compounds

ThOD: Theoretical oxygen demand

BOD biodegradation testing



- **Weight loss**
- **Morphology of surface**
- **Mechanical properties**
- **Plastisphere analysis**

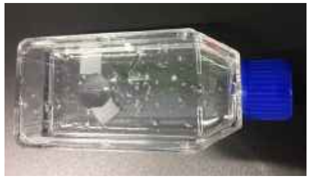
Tank experiment

E4 Optimization of *in vitro* test condition

① Biofilm development for obtaining plastic degraders

Laboratory (4 months)

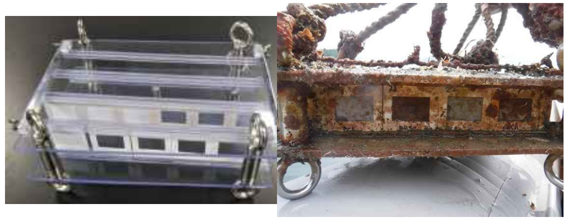
PBSA and PBAT films were put in sea water of 4 sites.



In the sea (5 months)

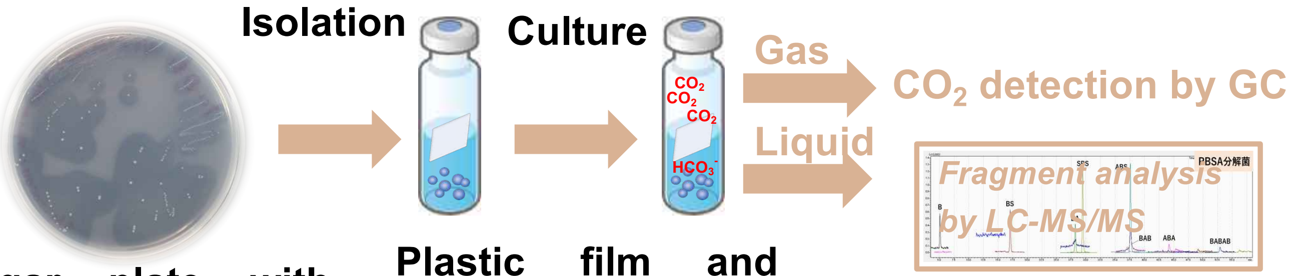
(Hiroshima, 10 m-depth)

Films of PBSA, PBS, PBAT, and PCL with film mounts were fixed in the fixture.



●PBSA/PBAT degraders (230 strains) were isolated from plastisphere.

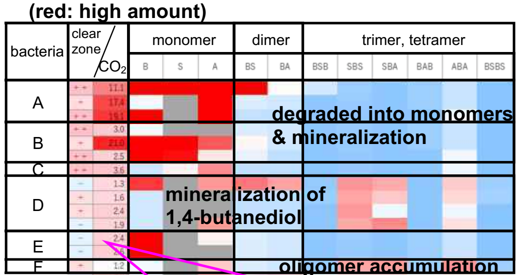
② Degradation property analyses of degraders on each material and degrader selection for rapid test method



Agar plate with emulsified plastics.

Plastic film and bacteria in artificial seawater.

Biodegradation properties of bacteria A-F (red: high amount)



clear zone development: -, CO_2 production: +

●Degradation property of each degrader has been analyzed by clear zone development, CO_2 production and accumulated degradation products.

To create a clean earth in the future

Thank you for your attention.

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RESEARCH & DEVELOPMENT PROGRAM