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שם המחלקה והמוסד המגישים:
המכון הגיאולוגי

יציבות והשפעות הידרולוגיות של הידרופוביות קרקעות מזוהמות בנפט באגן עברונה

Persistence and hydrological effects of oil-induced soil hydrophobicity in the Evrona basin

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תודות: ברצוננו להודות לרשות הטבע והגנים: ד"ר אסף צהר אקולוג מחוז דרום, ד"ר טל פולק האקולוגית המרחבית ועודד סהר הפקח האזורי, על שאפשרו לנו לעבוד בשמורה. לפרופ' יוני לרון, פרופ' עפר דהן ומשגב גולדנברג מאוניברסיטת בן גוריון על שיתוף הפעולה המלא בסקר הקרקע, בבחירת אתר הניסוי ובהגשת היתר המחקר. ליעל יעקובי מהמכון הגיאולוגי על אנליזות גודל הגרגר, לאורי מליק מהמכון הגיאולוגי על העזרה בהקמת תחנת הניטור, ולרמי מדמון, יעקב רפאל ודניאל צבי מהמכון הגיאולוגי על העזרה בשדה.

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תקציר

בלילה שבין ה-3 ל-4 בדצמבר 2014 דלפו כ-5 מיליון ליטרים של נפט גולמי כתוצאה מביקוע צינור ליד צומת באר אורה. הנפט התפשט לכיוון דרום מזרח לאורך ערוצי הפזרות של נחל רחם תוך שהוא גולש לתוך שמורת עברונה הסמוכה. כתוצאה מהמגע עם הנפט, שינו הקרקעות שזוהמו את תכונותיהן והפכו להידרופוביות ודוחות מים. בהמשך התברר כי קרקעות באתר סמוך, שזוהם בנפט עוד בשנת 1975, מאופיינות גם הן ברמת הידרופוביות גבוהה שלא נעלמה על אף שעברו למעלה מ-40 שנה מאירוע הדליפה. קרקעות הידרופוביות נוטות ליצור קרומים אטומים, להקטין חידור מים לקרקע, להגדיל יצירת נגר עילי, להגביר תופעות זרימה מועדפת, ולפגוע בפוריות הקרקע וביכולת צמחים לנבט ולהתבסס. ואכן, בסקרי שדה ובניסויי מעבדה נתגלה כי בקרקעות שזוהמו בשני אירועי הדליפה בעברונה ישנה האטה דרסטית בקצב נביטת עצי שיטה. לאור עדויות אלו, נראה כי להידרופוביות הקרקע ישנה השפעה גדולה על זרימת המים, והסעת המזהמים באגן, ותפקיד מרכזי בטיפול בזיהום ובשיקום הצמחייה בעברונה.

תכונות הקרקעות ההידרופוביות, המנגנונים המביאים להיווצרותן והשפעותיהן על מאזן המים ועל פוריות הקרקע נחקרו רבות בעשורים האחרונים. רב העבודות התמקדו בהידרופוביות שמקורה בחומרים טבעיים, בשריפות יער ובהשקיה בקולחים בעוד רק מקצתן עסקו בהידרופוביות שמקורה בזיהומי נפט. מבין עבודות אלו, הרב המוחלט בחנו קרקעות הנמצאות באקלים ממוזג. למיטב ידיעתנו, לא נחקרו קרקעות הידרופוביות שמקורן בזיהום נפט ונמצאות באזורים צחיחים כדוגמת אזור עברונה. מכיוון שידוע שטמפרטורה גבוהה ותכולת רטיבות נמוכה מעודדות הידרופוביות, יש לצפות כי לאקלים תהיה השפעה על מידת ההידרופוביות וכתוצאה מכך על תכונות הקרקע.

מטרות המחקר הן אפיון יציבות ועמידות ההידרופוביות בקרקעות המזוהמות בעברונה, מדידת תכונות הקרקעות ההידרופוביות וחקירת התנאים המיטביים לפירוק הנפט בקרקע ודעיכת ההידרופוביות. המחקר משלב ניסויי שדה למדידה רציפה של חידור וזרימת מים בקרקע, ניסויי מעבדה למדידת תכונות הקרקע, מעקב אחרי קצב פירוק הנפט ודעיכת ההידרופוביות בקרקע תחת תנאים סביבתיים שונים וכימות ואפיון המצאות אוכלוסיות חיידקים מפרקי נפט בקרקע.

בשנה הראשונה של המחקר נעשה סקר קרקע באגן על מנת לאפיין את מרקמה, הוקמה תחנת ניטור למדידת רטיבות קרקע, טמפרטורה ומוליכות חשמלית בערוץ מזוהם ובערוץ סמוך נקי, והועמד ניסוי הדגרה בקרקעות מזוהמות על מנת לעקוב אחר פירוק הנפט ודעיכת ההידרופוביות בתנאים שונים.

בשנת המחקר השנייה הוקמה תחנת ניטור שנייה במעלה האגן ונמשך הניטור בתחנה במורד האגן. כמו כן, המשכנו את ניסוי ההדגרה שהחל בשנת המחקר הראשונה. עם סיומו, אופיינה תאחיזת המים של הקרקעות ונבדקה אוכלוסיית החיידקים מפרקי הפחמימנים. בשנת המחקר השלישית נמשך ניטור רטיבות הקרקע

בשתי התחנות ונעשו ניסויים לאיפיון האוכלוסייה הטבעית של מפרקי הפחמימנים בקרקע וכן למציאת חיידקים בעלי פוטנציאל להפרשת חומרים פעילי שטח ולאפיון חומרים אלו.

בשתי תחנות הניטור, מעקב אחרי חידור וזרימת המים בקרקע התבצע בשני ערוצים סמוכים הרחוקים כ- 10 מטר אחד מהשני- אחד מזוהם ואחד נקי. בכל אחד מהערוצים נחפרה שוחה בעומק של כמטר שמונים לרוחב הערוץ. בקיר כל שוחה הותקנו 12 חיישנים (3 מסוג TDR ו-9 מסוג TDT) המודדים את רטיבות הקרקע, הטמפרטורה והמוליכות החשמלית החל מעומק של 7 ס"מ ועד לעומק של מטר ועשרים. צפיפות החיישנים גדולה יותר בעומקים הרדודים ויורדת בהדרגה עם העומק. באחת התחנות הותקן בנוסף מד גשם. על מנת לאפיין את חתך הקרקע, נלקחו דגימות קרקע לא מופרות משני חתכים אנכיים בקיר השוחה הממוקמים בשני קצותיו של הערוץ על מנת למדוד את תכונות הקרקע (צפיפות, פורוזיות, רטיבות, גודל גרגר). דגימות קרקע נוספות נלקחו משני החתכים ובין החיישנים על מנת למדוד את תכולת הנפט ואת הידרופוביות הקרקע. לאחר התקנת החיישנים, השוחות כוסו בקרקע על מנת לשמור על זרימת הקרקע לאורך הערוץ ולצמצם פגיעה בנוף. המדידות מבוצעות אוטומטית כל רבע שעה. הנתונים מאוחסנים באוגר נתונים ומשודרים מדי יום לשרתי המכון הגיאולוגי.

הקרקע בערוצים מורכבת משכבות חול וגבס. בהשוואה לקרקע במורד האגן, הקרקע במעלה האגן יותר גסה, מכילה אחוז גדול יותר של אבנים ושכבות הגבס בה פחות מסיביות. כמו בתחנה במורד, גם בתחנה במעלה, ערכי צפיפות השדה והפורוזיות שהתקבלו מדגימות הקרקע הלא-מופרות אופייניות לקרקעות חוליות, אם כי אחוז החומר הגס בתחנה במעלה גדול הרבה יותר. הרטיבות שנמדדה מהדגימות הלא מופרות, נמוכה מאוד כתוצאה ממוזג האוויר הצחיח והחם וקצב ההתאדות הגבוה.

במהלך המחקר היו ארבעה אירועי גשם משמעותיים, בסוף אוקטובר 2018, בתחילת פברואר 2019, בסוף פברואר 2020 ובאמצע מרץ 2020. שלושה מהאירועים הובילו להצפת השמורה וכולם התרחשו חידור מים לקרקע. עבור אירועי הגשם שהתרחשו באוקטובר 2018 ובמרץ 2020, תוצאות ניטור הרטיבות במורד מראות כי הרטיבויות בערוץ המזוהם היו קטנות משמעותית מהרטיבויות בערוץ הנקי וסך חידור המים במהלך האירוע היה קטן פי 4.5-4 בערוץ המזוהם ביחס לערוץ הנקי. יתרה מזו, בארבעת האירועים התקבלו בערוץ המזוהם דפוסי הרטבה לא רגולריים המעידים על פירוס רטיבות לא הומוגני ועשויים להצביע על זרימה מועדפת בקרקע ההידרופובית. מדידות של התכונות הידרוליות הלא רוויות של הקרקעות הצביעו על הבדלים בתכונות הקרקע המזוהמת והנקייה הכוללים תאחיזת מים פחותה בקרקע המזוהמת ומוליכות רוויה גדולה יותר. הבדלים אלו עשויים להסביר, לפחות חלקית את השוני בדפוסי הזרימה בין שני הערוצים. בתחנה במעלה לא התקבלו נתונים חד משמעיים עקב כך שהזרימה בערוץ המזוהם היתה משמעותית קטנה מהזרימה בערוץ הנקי.

בשתי התחנות, הטמפרטורות שנמדדו בערוץ המזוהם היו באופן עקבי גבוהות מאלו שנמדדו בערוץ הנקי. ההבדלי הטמפרטורות היו יותר גדולים יותר במורד, כנראה משום שהזיהום שם היה כבד יותר. הבדל זה עשוי לנבוע מהצבע הכהה של הקרקע המזוהמת או מהתאדות פחותה מקרקעות אלו.

על מנת ללמוד מהם התנאים האופטימליים לפירוק הנפט והפחתת ההידרופוביות הקרקע ועל מנת לחקור את הקשר בין זיהום הנפט להידרופוביות, הועמדו ניסויי הדגרה תחת תנאים שונים ובוצע מעקב אחרי קצב פירוק הנפט ודעיכת ההידרופוביות בקרקע. לצורך הניסויים נלקחו קרקעות מזוהמות מאתר הדליפה של 2014 ומאתר הדליפה של 1975. בנוסף, לצורך השוואה, נלקחה קרקע נקייה מערוץ סמוך לאתר הדליפה של 2014. סך הכל הועמדו 10 טיפולים: 7 עם הקרקע המזוהמת מ-2014 ו-3 עם הקרקע המזוהמת מ-1975. בקרקע מ-2014 נבדקו: קרקע ברטיבות של 20% רוויה, קרקע ברטיבות של 50% רוויה, קרקע ב-20% רוויה בתוספת נוטריינטים, קרקע ב-20% רוויה בתוספת חומר פעיל שטח (חפ"ש), וקרקע ב-20% רוויה בתוספת נוטריינטים וחפ"ש. כטיפול ביקורת שימשה קרקע ביובש אוויר. כאמור, קרקע נקייה מהאתר שמשה להשוואה. בקרקע מ-1975 נבדקו: קרקע ביובש אוויר כביקורת, קרקע ב-20% רוויה וקרקע ב-20% רוויה בתוספת נוטריינטים. תערובת הנוטריינטים שהוספו בניסוי הכילה חנקן, אשלגן וזרחן שנועדו לספק לחיידקים חומרים מזינים ללא תוספת פחמן. רמנוליפידים שמשו כחפ"ש בניסוי בשל תאימותם הביולוגית והניסיון הרב שנצבר בשימוש במוצרים אלה. מדי חודש, נלקחו מכל טיפול דגימות קרקע לבדיקות ההידרופוביות ותכולת נפט.

ההידרופוביות הקרקע נמדדה בשלוש שיטות: מדידת זווית מגע של טיפת מים, זמן חידור טיפה, ומולריות של טיפת אתנול. בשיטה הראשונה מצולמת טיפת מים המונחת על משטח קרקע וזווית המגע של טיפת המים עם הקרקע מחושבת מתוך התמונה. בשיטה השנייה מוערכת דרגת ההידרופוביות מתוך הזמן בו לוקח לטיפת מים לחזור את פני הקרקע. ככל שזמן זה גדל - ההידרופוביות גדלה. בשיטה השלישית מונחות על פני הקרקע טיפות של תמיסות של אתנול ומים בריכוזים שונים ונמצא ריכוז האתנול המינימלי בו הטיפה חודרת את פני הקרקע תוך חמש שניות. ככל שריכוז האתנול גבוה יותר, הקרקע יותר ההידרופובית.

תכולת והרכב הנפט נמדדו באמצעות גז כרומטוגרף (GC-MS) ריכוז סך מרכיבי הנפט (TPH) נמדד במעבדה חיצונית מוסמכת. כאינדיקציה לפירוק הנפט שמשו היחסים בין פריסטאן Pr ופיטאן Ph (פחמימנים בעלי שרשראות מסועפות בהם 17 ו-18 פחמנים, בהתאמה), לבין אלקנים (פחמימנים בעלי שרשראות ישרות). ככלל, חיידקים ייטו לפרק קודם פחמימנים בעלי שרשראות ישרות ולכן עלייה ביחס בין פחמימנים בעלי שרשראות מסועפות לישרות מעידה על פירוק. בנוסף נבדק גם היחס בין אלקנים בעלי משקל נמוך L (המכילים בין 14 ל-21 פחמנים) לבין אלקנים בעלי משקל גבוה H (המכילים בין 22 ל-34 פחמנים). כיוון שחיידקים נוטים לפרק קודם אלקנים קצרים, ירידה ביחס L/H מצביעה על פירוק.

תוצאות ניסויי ההדגרה מצביעות על כך כי כאשר הוספו לקרקע מים ב-20% רוויה או מים וחפ"ש או נוטריינטים או שניהם, נצפה פירוק נפט במקביל לירידה בהידרופוביות. לאחר שנה וחצי של הדגרה, ריכוז

הפחמימנים וההידרופוביות באותם טיפולים היו דומים. עם זאת, קצב פירוק הנפט וקצב הירידה בהידרופוביות השתנו מטיפול אחד לשני. בקרקע המזוהמת מ-2014, הפירוק המהיר ביותר התרחש בקרקע עם הנוטריינטים והחפ"ש. לאחר חודשיים, זמן חידור טיפה ירד מלמעלה שעה לכ-8 דקות, מולריות טיפת אתנול ירדה למחצית מערכה ההתחלתי, ערך- $Pr/C17$ עלה מ-0.41 ל-38, ערך $Ph/C18$ עלה מ-0.56 ל-42 ויחס L/H ירד מ-0.9 ל-0.36. כאשר הוספו לקרקע רטובה נוטריינטים בלבד, הפירוק התחיל מעט מאוחר יותר, אולם לאחר מספר חודשים רוב הפרמטרים השתנו לאלו של הקרקע שהוספו לה נוטריינטים וחפ"ש. בקרקע הרטובה עם החפ"ש הפירוק ודעיכת ההידרופוביות החלו רק אחרי 3-4 חודשים. כאשר לקרקע הוספו מים בלבד, קצב הפירוק היה איטי מאוד ונצפה רק לאחר כ-5 חודשים. נמצא כי בקרקע שהייתה ברוויה של 20% פירוק הנפט והירידה בהידרופוביות היו גדולים מבקרקע שהורטבה לדרגת רוויה של 50%. נתון זה מראה כי אין צורך להרטיב את הקרקע לדרגת רוויה גבוהה על מנת שיתרחש פירוק טבעי.

בהשוואה בין הקרקע המזוהמת מ-1975 לקרקע מ-2014, הנפט מ-1975 הכיל פחות פחמימנים קצרים, כנראה כתוצאה מפירוק שהתרחש במשך 40 השנים שחלפו מאז אירוע הדליפה. כאשר הוספו לקרקע מים ונוטריינטים קצב הפירוק ודעיכת ההידרופוביות בקרקע מ-1975 היו מהירים יותר וכבר אחרי חודש זמן חידור טיפה ירד ממעל שעה לכ-16 דקות, יחס $Pr/C17$ עלה מ-0.3 ל-4, וערך $Ph/C18$ עלה מ-0.45 ל-20.6. ייתכן שהסיבה לכך היא שהאוקלוסייה המיקרוביאלית באתר זה מותאמת יותר לפירוק נפט בעקבות החשיפה הארוכה לנפט. תוצאות בדיקת סך מרכיבי הנפט (TPH) בתחילת ניסוי ההדגרה ולאחר שנה וחצי הצביעו כי בקרקע שטופלה בתוספת מים ונוטריינטים חלה ירידה של 82% ו-40% בתכולת הנפט בקרקע מ-1975 ומ-2014. תוצאות אלו מצביעות על כך שדי בתוספת מים על מנת לגרום לפירוק נפט משמעותי, הוספת נוטריינטים וחפ"ש מגבירה את קצב הפירוק אבל לא את ריכוז הנפט הסופי. ייתכן שבסוף ניסוי ההדגרה גורם אחר (למשל מיקרו נוטריינט) הגביל את המשך פירוק הנפט. לבסוף יש לציין כי למרות הירידה בהידרופוביות, הקרקע עדיין דוחת מים ומאופיינת ברמת הידרופוביות גבוהה, על כל המשמעויות השליליות הנלוות לכך.

לאחר סיום ניסויי ההדגרה נמדדה תאחיזת המים בטיפולים השונים באמצעות שיטת ה-Dew point בייבוש ובהרטבה. בעוד לא נמצאו הבדלים משמעותיים בתאחיזת המים בין הטיפולים השונים, נראה כי בטיפולים שהכילו חפ"ש ו/או נוטריינטים התגלתה היסטריזה פחותה ביחס ליתר הטיפולים. אכן, ידוע כי חומרים פעילי שטח מפחיתים את ההיסטריזה. ייתכן שבטיפולים בהם הוספו נוטריינטים הופרשו חפ"שים על ידי חיידקי הקרקע.

בדיקות נוספות שבוצעו על דגימות הקרקע מניסוי ההדגרה נועדו לאפיין את אוכלוסיית מפרקי הנפט הטבעית ולבודד חיידקים בעלי יכולת הפרשת חומרים פעילי שטח העשויים לסייע בפירוק הנפט. הערכת מספר החיידקים מפרקי הפחמימנים התבצעה הן בשיטת הערכת המספר המסתבר ביותר (MPN, Most

Probable number) והן בשיטות מולקולריות (qPCR), בשתי השיטות נמצא כי קיימים חיידקים מפרקי פחמימנים בכל דוגמאות הקרקע, גם בקרקע הנקייה.

בהמשך בודדו מהקרקע שמונה זני חיידקים המסוגלים להפריש חומרים פעילי שטח. נמצא כי כולם שייכים לסוג פסאודומונס וקרובים ברצף הגנטי שלהם. פעילות החיידקים אופיינה על ידי מספר בדיקות המאפיינות את יכולת החיידקים להפריש חפ"שים ואת עצמת החפ"שים. מתוך שמונת החיידקים, נמצא כי לרשה פוטנציאל גבוה לשמש לפירוק פחמימנים טבעי.

תוצאות המחקר מצביעות על כך שלהידרופוביות השפעה דרסטית על הזרימה ועל ההסעה באגן. הקרקעות ההידרופוביות עוצרות חידור מים ונוטות לזרימה מועדפת. כתוצאה מכך צפויה עלייה בנגר העילי ובסחף, מחד, וירידה במים הזמינים לצמח, מאידך. על כן יש ללמוד טוב יותר את השפעת ההידרופוביות הקרקע ברמת האגן וההשפעה על מאזן המים ותנועת מזהמים. מסקנה נוספת של המחקר היא שניתן לפרק אחוז גבוה מהנפט תוך שמירה על רטיבות נמוכה יחסית והוספת נוטריינטים בלבד. עם זאת, גם לאחר פירוק של כמות גדולה של המזהם, ההידרופוביות הקרקע נותרת גבוהה, ולכן יש לנטר גם את ההידרופוביות הקרקע ולא להסתפק במדידת תכולת המזהם. לבסוף, נמצא כי הקרקע הטבעית מכילה חיידקים מפרקי נפט וכן חיידקים בעלי יכולת הפרשה של חומרים פעילי שטח היכולים לסייע לפירוק הנפט. ששה מחיידקים אלו בודדו ונמצאו כבעלי פוטנציאל גבוה מבחינת הפרשת חומרים פעילי שטח. דרושה עבודה נוספת על מנת לראות אם ניתן להשתמש בהם לפירוק טבעי של זיהומי נפט.

Abstract

Soil hydrophobicity has a central role in oil containment and site remediation in the Evrona 2014 and 1975 spill sites. In this study, we combine field monitoring, laboratory incubation experiments, measurements of soil hydraulic properties and microbiological assays to investigate the persistence and hydrological effects of oil-induced soil hydrophobicity in Evrona.

Water contents and temperatures were monitored in adjacent polluted and contaminated streams at two sites, one at the upstream of the basin and the other in its downstream. The soils in the basin are predominantly sandy with the upstream section having larger fraction of coarse material. During the two-year monitoring period four substantial rain events took place. Results of the water content measured during these events show a substantial decrease in infiltration in the contaminated stream and irregular patterns indicating preferential flow in the polluted soil. Unsaturated hydraulic properties of clean and contaminated field samples show and an increased saturated hydraulic conductivity and decreased water retention in the contaminated soils compared to the clean ones. Temperatures in the contaminated streams were found to be consistently higher by up to 1 °C relative to the clean stream. This difference may result from the darker color of the oil-polluted soil or from lower evaporation from the contaminated soil.

Incubation experiments were conducted with soils from the 2014 and 1975 polluted sites. Soils were treated with the addition of water alone, water and nutrients, water and surfactants, and a combination of water, surfactants and nutrients. The results of the incubation experiment showed a concomitant and significant decrease in hydrophobicity and hydrocarbon content in soils to which water and nutrient, surfactants or water to 20% saturation were added. At the end of the incubation experiment, the level of hydrophobicity and hydrocarbon was similar in all of these treatments. The rate of reduction was, however, different; The fastest degradation was observed in soil which was spiked with both nutrients and surfactants, while in 50% saturation soil, degradation and hydrophobicity reduction were minor and very slow. Degradation and hydrophobicity reduction in soil saturated to 20% was higher than in soil saturated to 50% showing that wetting the soil to high saturations is not needed for bioremediation. The 1975 soil treated with addition of nutrients showed a faster response compared to the 2014 soil, possibly as a results of a more adapted microbial community in the 1975 site. Overall, during 1.5-year incubation, the total petroleum hydrocarbon of the soil treated with nutrients decreased by 40% in the 2014 soil and by 82% in the 1975 soil. This shows that addition of even water alone is sufficient to trigger substantial bioremediation, which is accompanied by hydrophobicity decrease. Yet, it is important to note that the soils are still severely hydrophobic and water repellent. We therefore conclude that when conducting bioremediation treatment, it is not enough to monitor the content of hydrocarbons, but soil hydrophobicity should be monitored as well.

Abundance of oil degraders was evaluated by qPCR and MPN, the results showed that these bacteria are found in all treatments, even in the clean soil. Surprisingly, the 1975 soils did not show higher numbers of bacteria relative to the 2014 soils, similarly no clear effect was shown by any of the treatments.

Finally, biosurfactant-producing strains were isolated and their activity was evaluated. Eight high-potential strains, which belong to the *Pseudomonas* genus, were isolated. Out of the eight isolated strains, six were found to be particularly efficient in biosurfactant production and thus exhibit the potential to be used for oil bioremediation and should be further evaluated in future research.

מילות מפתח : עברונה, דליפת נפט, הידרופוביות, דחיית מים, תכונות הידרוליות, חידור, פירוק ביולוגי, האיזור הבלתי רווי, פירוק טבעי, תכולת רטיבות

ביצוע מול תכנון	משימה: סקר ספרות בוצע משימה: העמדת ניסויי הדגרת קרקע מזוהמת תחת תנאים שונים על מנת לעקוב אחרי פירוק הנפט והידרופוביות הקרקע בוצע. משימה: ביצוע סקר קרקע לאפיון מרקם ומבנה הקרקע באגן עברונה בוצע משימה: התקנת תחנות לניטור רטיבות קרקע, טמפרטורה ומוליכות חשמלית בערוצים מזוהמים ונקיים. בוצע משימה: ניתוח רטיבות בחתכים המנוטרים באירועי גשם. בוצע. במהלך השנתיים האחרונות נרשמו ארבעה אירועי גשם עיקריים. משימה: מדידת תכונות הידרוליות של קרקעות נקיות ומזוהמות. בוצע. תאחיזת המים של הקרקע מהטיפולים השונים נמדדה במכשיר WP4C. בנוסף תאחיזת מים ומוליכות הידרולית של הקרקעות הנקיות והמזוהמות נמדדו בשיטת ההתאדות. משימה: בדיקת המצאות חיידקים מפרקי נפט בקרקעות המזוהמות והנקיות ופוטנציאל הפרשת חומרים פעילי שטח. בוצע.
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Introduction

On December 3rd 2014, about 5000 cubic meters of crude oil leaked from a breached pipeline west of the B'eer-Ora junction, spread to the southeast, flushing the braided streams of Nahal Raham (Figure 1). The oil polluted a gross area of over 10 km², much of it located inside the Evrona nature reserve (Samet, 2015). The penetration depth of the oil into the soil in the nature reserve was between a few millimeters to a maximum of 30 cm (Samet, 2015; LDD, 2015). In the few first days after the spill, several emergency measures such as excavation of storage pools, construction of dams and oil pumping were taken to prevent the oil from spreading further into the nature reserve, across the Jordanian border and into the Gulf of Eilat. Additionally, about 50,000 tons of contaminated soil were excavated and transferred to dedicated treatment facilities (Samet, 2015; Shmuel Neaman Institute, 2015). It is estimated that around 700,000 liters of oil are still on site. Since the area is ecologically vulnerable, it was decided not to continue with invasive operations, but instead examine alternatives of in-situ treatments.

Preliminary results from studies conducted in the Evrona nature reserve during the year after the oil spill indicate that the oil altered the soils' physical properties such that the polluted soils turned hydrophobic and water repellent (Tunis and Kisra, 2016, Gordon et al., 2018). Such soils repel water, decrease soil aeration and infiltration, and increase surface runoff (Dekker et al., 2005). These changes have negative effects on the germination and growth of plants, as was recently demonstrated in a nearby oil spill site which occurred in 1975 (Nothers et al., 2017; Tran et al., 2018). In this site, no active remediation was conducted, and residues of oil are still present at the top 1.5 m of the soil profile (LDD, 2015). Although more than 40 years have passed since the pollution event, the polluted soils are still as severely water repellent as the soils in the 2014 site (Gordon et al., 2018). It was found that the density of young trees in the polluted site is lower than in neighboring non-polluted areas (Nothers et al., 2017). Additional lab experiments showed that germination is considerably hindered in the polluted soils (Nothers et al., 2017; Tran et al., 2018). These results indicate that soil hydrophobicity plays and will play a key role in the containment and remediation of the recent contamination in Evrona.

Although soil hydrophobicity is studied intensively in recent decades in the context of natural hydrophobicity, forest fires and wastewater irrigation, relatively little is known of oil-induced soil hydrophobicity, particularly in hyper-arid areas.

Objectives:

The primary objective of this research is to **study the persistence and hydrological effects of oil-induced soil hydrophobicity in the hyper-arid Evrona basin** and to **investigate the relation between hydrocarbon content and the severity of hydrophobicity**. To reach this, we set the following three tasks,

1. Study how oil pollution and induced hydrophobicity affects infiltration depth, preferential flow and transport in soils by in-situ monitoring the water content, temperature and electrical conductivity in clean and polluted soil sections and by sampling the soil for hydrocarbon content.
2. Investigate how oil-induced hydrophobicity affects soil hydraulic properties by measuring the hydraulic properties of undisturbed clean and polluted soil samples.
3. Assess the natural attenuation of oil and the decrease in the degree of hydrophobicity under different environmental conditions, and study the relation between the content and composition of hydrocarbons and the severity of hydrophobicity and its adverse effects. This objective will be achieved by conducting controlled incubation experiments under different water contents and nutrition conditions while monitoring evolution of hydrocarbon content and composition and soil hydrophobicity over time.
4. Evaluate the microbial populations which can degrade hydrocarbons and produce biosurfactants and/or emulsifiers.

Literature survey

The Evrona playa – geomorphological and hydrological settings

The Evrona playa is located in a 10 km long and 0.5-2 km wide basin at the southern Arava valley, east of road 90 and about 15 km north to Eilat (Figure 1) (Amit et al., 1999; Segev, 2016). Nahal Raham and Nahal Evrona drain the region towards the Gulf of Eilat (Amit et al, 1999). These two wadis form alluvial fans near their outlets, with typical braided river morphology of streams and terraces; further downstream the elevation difference between streams and terraces decreases. Three distinct zones can be identified: 1) the central, barren zone; 2) the transitional zone, and 3) the outer vegetated zone with halophytic vegetation (Amiel and Friedman, 1971). The soils in the playa are saline, and vary between fine desert alluvial soils in the vegetated areas to clay loam alluvial sterile Solontchaks in the barren zones (Amiel and Friedman, 1971; Dan, 1981). In general, the alluvial sediments are arranged according to concentric belts, with clay-rich sediments in the lowest part of the playa, generating a pattern of a decreasing grain size towards the downstream (Amit et al., 1999, Crouvi, 2002, Segev, 2016). The depth of the alluvium thickens towards the southeast from tens of meters to a few hundred meters (Segev, 2016). In addition to the alluvial soils, gypsum and salt crusts are common in the central part of the playa.

The climate in the region is extremely arid, with mean annual rainfall of ~30 mm, which is usually concentrated in one or two rain events. The potential evaporation is 2600 m/year, the average temperature is 25°C and the mean relative humidity is around 40-45%. The surface temperature is very high and can reach 50°C at summer days (Amit

et al, 1999). The playa is a unique ecosystem which hosts a diverse population of plants and animals. Among these are the northeast doom palms, the largest herd of Dorcas gazelles in Israel and a large density of Acacia trees.

The hydrological system in the region consists of brackish aquifers. The regional aquifer west of road 90 is phreatic with water level ranges between 45 m and 60 m below the surface, whereas below the nature reserve it is confined with the water level depth ranging between 10 m and 17 m (Bein et al., 1995; 2001). The flow direction in the aquifer is to the southeast, towards the Gulf of Eilat (Naor and Katz, 1988). Additional local shallow aquifer lies below the nature reserve (Zurieli, 2014). However currently it is not clear what its water level is.

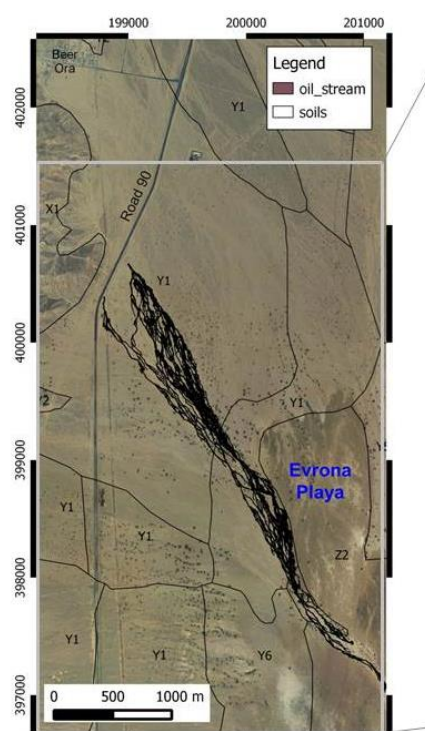


Figure 1: Location map of the Evrona playa and surroundings, showing the distribution of the 12/2014 oil spill (courtesy of the National Park Authority) and the soil types (courtesy of the Ministry of Agriculture). Soil type codes are as follows: Z2 – Solontchak, Y1 – Coarse desert alluvium, Y2 – Reg soil and coarse desert alluvium, Y5 – Sandy alluvium, Y6 - Reg sandy soil and eolian sand, X1 - Exposed bedrock.

Oil-induced soil hydrophobicity

The ability of soil to efficiently hold and transfer water and thus maintain its ecological functions is by a large extent the result of the wetting properties of water. Most natural soils are water-wet, i.e., water can easily wet the soil grains, forming a contact angle typically of $\sim 0^\circ$ between the grain-surface, water and air (Warrick, 2003). When oil is in contact with soil, it alters its physical properties by forming films of hydrocarbons on top of the soil grains (Adams et al., 2008; Marin Garcia et al., 2016). These films induce soil hydrophobicity and water repellency. In hydrophobic soils the contact angle between the water and soil grain increases up to a point where the soil surface is no longer water-wet and the contact angle is larger than 90° (Xiong et al., 2012).

Hydrophobic water-repellent soils are known to significantly affect the water balance and soil fertility by decreasing infiltration, increasing surface runoff and erosion, and impede plant growth and germination (Dekker et al., 2005; Li et al., 1997). Although the existence of hydrophobic and water repellent soils is long being known and was intensively studied (Dekker et al., 2005), most of the studied dealt with fire-induced, naturally-occurring, or wastewater related water-repellency (e.g., Doerr et al., 2000; Dekker and Ritsema, 1994; Wallach et al., 2005). Only a few studies focused on water repellency induced by oil pollution (Roy and McGill, 1998; Adams et al., 2008; Takawira et al., 2014). Of which, most studies focused on temperate cool climate while no studies were done under arid conditions (Takawira et al., 2014). In particular, climate and soil texture are expected to significantly affect the degree of water repellency as high temperature, low water content and coarse texture promote the generation of water-repellent soils (Adams et al., 2008, Buckzo et al., 2006). Thus, the sandy, dry soils in Evrona are especially prone to become hydrophobic and water-repellent.

As hydrophobicity alters soil structure and changes the spatial configuration of water within pores, it changes soil hydraulic properties (Takawira et al., 2014). Some studies report of hydrophobicity-induced alteration in soil hydraulic properties showing non-conclusive results. While most of the authors report of decreased water retention (Roy and McGill, 1998; Hyun et al., 2008; Takawira et al., 2014), others report of increased water retention, particularly under high suctions (Burckhard et al., 2000). Reduction of the saturated hydraulic conductivity with increasing hydrocarbon content was observed by Hyun et al. (2008), whereas conductivity increase was observed by Takawira et al. (2014). Preliminary results from Evrona spill-site also indicate a higher saturated hydraulic conductivity of the oil-polluted soil with respect to the clean soil (Tunis and Kisra, 2016, Gordon et al., 2018). Modifications of other properties such as porosity, shrinkage and bulk density were also reported (Caravaca and Roldan, 2003; Takawira et al., 2014) showing no consistent results. Water-repellent soils also tend to induce preferential flow, fingering and unstable wetting fronts (Dekker et al., 2005; Xiong et al., 2012). Such flow patterns may affect infiltration, plant survival, and solute transport. In particular, they can result in fast contaminant transport from the soil surface into the underlying groundwater.

Oil-induced hydrophobicity seems to be fairly persistent with soils being hydrophobic decades after the occurrence of the spill event (Roy and McGill, 1998). Some of these soils may have no obvious indication of contamination such as color or odor and have very low hydrocarbon content, but yet exhibit severe water repellency and hydrophobicity (Adams et al., 2008). Roy and McGill (1998), for instance, report the presence of hydrophobic soil at a site where crude oil contamination occurred over 50 years before sampling. Li et al. (1997) showed that soil treated with bioremediation which removed 50% of the hydrocarbon content remained almost as water-repellent as the contaminated soil. Further, plant growth and yield in the contaminated and remediated soils were drastically and almost equally reduced with respect to non-polluted soil. As noted before, also in Evrona, the soil in the 1975 spill site still exhibits some degree of hydrophobicity and a negative effect on plant germination is observed.

Natural attenuation of oil in polluted soils

Biodegradation is one of the significant natural attenuation processes which can result in contaminant removal or detoxification. The success of biodegradation depends on various physical and geochemical conditions in the contaminated site as well as on the nature and the amount of the contaminant present. Obviously, the presence of microorganisms with the appropriate metabolic capabilities is the basic requirement for biodegradation occurrence. In addition, several factors can affect the efficiencies and rates of hydrocarbons biodegradation in soil. These include temperature, pollutants concentration, pH, bioavailability of contaminants, oxygen and nutrients concentration, soil moisture and structure (Boopathy 2000; Sabaté et al. 2004; Torres et al. 2005; Iqbal et al. 2007).

Soil and groundwater bacteria use a variety of natural electron acceptors in the degradation process. The use of these final electron acceptors is based on energy transfer efficiency and availability. The most common inorganic electron acceptor is dissolved oxygen (DO). Presence of oxygen (>0.5 mg/L) is necessary for aerobic biodegradation. In saturated and in some of the unsaturated sites, subsurface oxygen is rapidly depleted and biodegradation rate is limited by the rate that oxygen is supplied to the microorganisms by diffusion. Once oxygen has been depleted, bacteria will preferentially use the next most efficient electron acceptor, usually nitrate (NO_3^-) or insoluble manganese (Mn^{4+}). After NO_3^- and Mn^{4+} have been depleted, the bacteria will use ferric iron (Fe^{3+}), followed by sulfate (SO_4^{2-}), and carbon dioxide (CO_2), respectively. During the reduction of these electron acceptors CO_2 , ammonia (NH_3), soluble manganese (Mn^{2+}) and iron (Fe^{2+}), sulfide (S_2^-), and methane (CH_4) are produced. Some of these nutrients could become limiting factors for biodegradation. Soils that have a pH of 6 to 8 usually promote optimal bacterial growth. The temperature range under which biodegradation occurs is quite broad (from 0 to 45°C). Usually biodegradation rate increases with temperature. A moderate level of soil moisture is necessary to support the growth of microbial populations. In addition to that, microorganism can only utilize petroleum hydrocarbons when the hydrocarbons are in the dissolved phase. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes $>$ branched alkanes $>$ small aromatics $>$ cyclic alkanes (Ulrici 2000). Resistance to biodegradation typically increases as the molecular weight of the hydrocarbon increases (Atlas 1981). Field studies at a crude oil spill site in India indicated that up to 75 percent of the hydrocarbons present could be biodegraded within a year if the proper geochemical and microbial consortia are present (Gogoi et al. 2003).

Microbial response to oil pollution was extensively investigated all over the world (Paisse et al., 2011; Silva et al., 2013; Genovese et al., 2014; Xu et al., 2018). The changes in the composition and diversity of the microbial community and oil degradation rates are affected by the availability of nutrients and hydrocarbons (Siles and Margesin, 2016).

Strong correlations were shown between the variation in microbial communities and the variation in organic carbon, clay content and soil moisture (Naveed et al., 2016). When exposed to crude oil, the diversity of the indigenous microbial community and the archaeal population significantly decreased in the deep Eastern Mediterranean Sea (Liu et al., 2017). Indigenous microorganisms may have the ability to adapt to oil contamination and the community structure can shift to a hydrocarbon-degrading-bacteria dominated structure. Oil residues were found to stimulate the richness of γ -Proteobacteria in the deep sea of the Gulf of Mexico (Hazen et al., 2010), while in another study, γ -Proteobacteria, β -Proteobacteria, and Actinobacteria were found to be major contributors at the initial stage of bio-degradation of oil-polluted saline soils (Gao et al., 2014). In the previous study conducted in soil samples from oil-contaminated soils in the Evrona basin, Proteobacteria was found to increase dramatically in contaminated soils compared to clean soils (Girsowicz et al., 2018). Moreover, the diversity of the microbial community was lower in the soils polluted in 1975 than in the soils polluted in 2014, indicating the persistence of adverse impact of the oil spill on the microbial populations. Hydrocarbon degrading bacteria are ubiquitous in the environment because of the presence of low-biotic originated hydrocarbons in soil and water (Rosenberg et al., 2013). Hydrocarbon degraders are found in virtually all-natural areas, although with significant variations in cell numbers. The ratio of hydrocarbon oxidizing bacteria to the total microbial population may change according to the time of sampling or to the extent of oil pollution. Several bacterial and fungal strains were isolated from oil-contaminated sites and proved to metabolize alkanes and PAHs during the biodegradation process. For instance, *Acinetobacter venetianus* 6A2 strain was found to have the ability to utilize n-alkanes ranging from C10 to C40 (Throne-Holst et al., 2006), while the indigenous strain *Gordonia* sp. CC-NAPH129- from a diesel-contaminated site was capable to utilize naphthalene as a sole carbon source and degraded 50 ml BSM-naphthalene medium in less than 24 hours (Lin et al., 2012).

Some studies investigated the hydrocarbon degraders in desert oil-polluted soil habitats. Muthukrishnan and Abed (2018) proposed that irrigation with nutrients significantly enhanced the biodegradation rates in oil-contaminated soils. This nutrient-amended oil-contaminated desert soil was dominated by the Gammaproteobacteria from which 50% of the isolated alkane-degrading strains belonged to *Pseudomonas*. A unique observation of oil-degrading bacteria from oil-polluted habitats in Kuwait was their potential to fix molecular nitrogen that gives them an advantage in nutrient-poor desert soil (Al-Awadhi et al., 2012). They found that the Actinobacteria was the specific hydrocarbon-utilizing bacterioflora in these terrestrial soil samples.

As already mentioned, hydrocarbon biodegradation is initiated by an oxidative process that involves the insertion of molecular oxygen into the compound. In turn, the aliphatic and aromatic compounds become more polar and less hydrophobic (Fig. 2). Therefore, alkane monooxygenase gene *alkB*, naphthalene dioxygenase gene *nahA* and phenol monooxygenase gene *dmpN* are typically used as indicators for the diversity and abundance of the corresponding alkanes and aromatics degrading bacteria (Pérez-de-Mora et al., 2011; Liu et al., 2015; Peng et al., 2015). The abundance of the *alkB* gene

was reported to be strongly correlated with counts of diesel-degrading bacteria by Most-probable-number (MPN) method in polar hydrocarbon polluted soils (Yergeau et al., 2009). The number of copies of the *alkB* gene was shown to be positively correlated with the total petroleum hydrocarbon (TPH) while the *Nah* gene was found to be negatively related to the concentration of total aromatic hydrocarbon (TAH) (Liu et al., 2015).

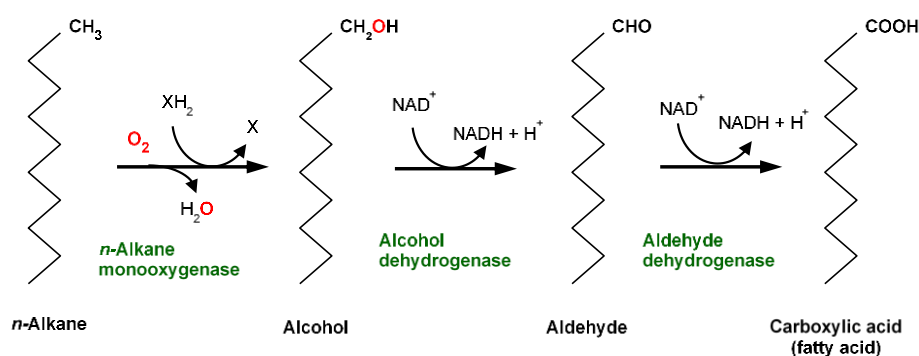


Figure 2: A general aerobic degradation pathway of n-alkane initiated by alkane monooxygenase that is coded by the *alkB* gene.

In summary, given the evidence of strong oil-induced soil hydrophobicity and long-term damage to vegetation in Evrona, the significant influence of soil-hydrophobicity on water flow and contaminant transport and the lack of knowledge of the effects of oil-induced soil hydrophobicity in arid environments, we conclude that the persistence and hydrological effects of soil hydrophobicity in Evrona should be studied to better assess water balance, oil fate and transport and the damage to vegetation in the polluted site and in the entire basin. The research is novel of several aspects; first it combines investigation of soil hydrophobicity, hydrocarbon content and oil-biodegrading bacterial population. Second, it is performed in a hyper-arid environment in which two spill events have occurred in the last 40 years. The combination of the hyper-arid climate and the reoccurring spill events presents a unique opportunity to study the effects of spill events in arid environments over time.

Biosurfactants in soil remediation

Biosurfactants are classified according to their molecular weight, into low-molecular-weight including glycolipids, phospholipids, and lipopeptides and into high-molecular-weight groups consisting of amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins or complex mixtures of these biopolymers (Ron and Rosenberg, 2002; Pacwa-Płociniczak et al., 2011). The low-molecular-weight biosurfactants are efficient in reducing the interfacial surface tension. The high-molecular-weight surfactants are less efficient in reducing interfacial surface tension but are efficient in coating the oil droplets and preventing their coalescence (Ron and Rosenberg, 2002). Biosurfactants are extensively used in the remediation of contaminated sites because of advantages such as being environmentally friendly,

having a good temperature, pH and ionic strength tolerance and biodegradability (Karlupudi et al., 2018). The low solubility and strong hydrophobicity of oil pollutants lead to poor bioavailability of these pollutants to hydrocarbon-degrading microorganisms in the bioremediation process. In concentrations lower than the critical micelle concentration (CMC), the surface tension of the solution decreases with the concentration of the biosurfactant. In concentrations above the CMC, biosurfactants will form micelles, bilayers, and vesicles (Fig. 3). Micelle formation reduces the surface and interfacial area of the hydrophobic part of the biosurfactant that is in contact with the solution and increases the solubility and bioavailability of hydrophobic organic compounds.

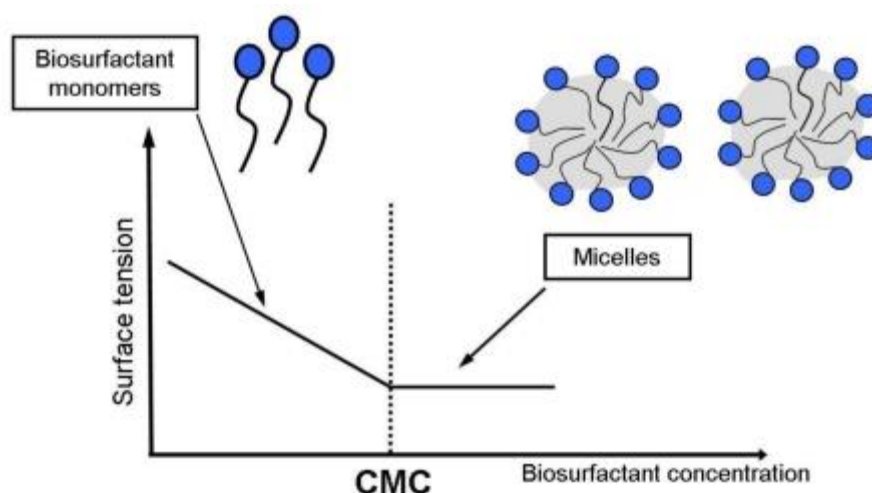


Figure 3: The relationship between biosurfactant concentration, surface tension, and formation of micelles (Whang et al., 2008).

Two major mechanisms through which biosurfactants enhance hydrocarbon bioremediation include increasing the solubility of hydrocarbons and increasing the bioavailability of hydrophobic compounds (Ron and Rosenberg, 2002). A decrease of the cell surface hydrophobicity was demonstrated in *Acinetobacter calcoaceticus* RAG-1 in the presence of biosurfactant. that the biosurfactant enables the cell to attach to oil droplets and utilize long-chain alkanes (Ron and Rosenberg, 2002). Many studies have reported enhanced hydrocarbon removal achieved by rhamnolipids, a common biosurfactant, produced by *Pseudomonas aeruginosa*, which was found to stimulate uptake of hydrophobic compounds (Noordman and Janssen, 2002). For instance, Perfumo et al. (2006) reported that *Pseudomonas aeruginosa* AP02-1 isolated from the hot spring environments synthesized a mixture of rhamnolipids with high emulsifying activity when grown on glycerol. In another study, a novel rhamnolipid biosurfactant-producing bacterium *Pseudomonas aeruginosa* strain NY3 was shown to have the ability to enhance the degradation of high molecular weight PAHs using its biosurfactant (Nie et al., 2010).

As far as we know, most of the studies related to oil-induced soil hydrophobicity were conducted in temperate and humid environments (Adams et al., 2008; Takawira et al. 2014), while the oil spill in Evrona is the only example of oil-induced soil hydrophobicity in hyper-arid climate (Gordon et al., 2018; Stavi and Rosenzweig, 2019). Gordon et al. (2018) reported the persistence of in-situ soil hydrophobicity over

42 years, as indicated by the high water drop penetration time and contact angle in the 1975 polluted site which were equivalent to or even higher than in the 2014 site.

There are only a few studies related to the oil pollution-induced alternations in soil hydraulic properties in the hyper-arid Evrona basin (Gordon et al., 2018; Stavi and Rosenzweig, 2019). Gordon et al. (2018) reported that the saturated hydraulic conductivity of field samples was higher in the contaminated soil than in the clean soil. Increased penetration resistance in the contaminated soils was also measured in both the 1975 and 2014 sites compared to corresponding non-contaminated soils (Gordon et al., 2018). Stavi and Rosenzweig (2019) investigated the effect of a 10cm tillage on the hydrological properties of the polluted soil. They found the effects were limited. At the ground surface, the penetration resistance was significantly decreased after the tillage while the hydraulic conductivity was only slightly and non-significantly increased. No beneficial effect was observed in deeper soil layers. Therefore, more research is needed to evaluate the potential implications of oil pollution on the soil properties and to improve hydrological properties.

Although the indigenous microbial populations in oil-contaminated marine environment have been comprehensively investigated (Kimes et al., 2014; Yergeau et al., 2015; Ribicic et al., 2018; Miller et al., 2019), knowledge of microbial response to oil contamination and to the addition of water and nutrients is still limited, especially in the hyper-arid terrestrial systems. Thus, research is needed to better understand the microbial populations in oil-contaminated sites in drylands.

Methods

Soil survey

As a preliminary step for choosing the location of the monitoring station, we conducted a soil survey where soil was sampled in 20 points along the contaminated stream to characterize the soil texture (Figure 4). In each point soil was typically sampled in 5 depths starting from the soil surface up to a depth of 1 m. The survey was conducted in full collaboration with the group of Prof. Jonathan Laronne and Prof. Ofer Dahan from Ben Gurion University. Soil grain size distribution and texture were determined by laser diffraction using Mastersizer (Malvern, UK), yielding the complete range of soil grain size, from sub-microns to 2 mm (Crouvi et al., 2008).

Hydrological field monitoring

Two monitoring stations in two sites (upstream and down stream, see Figure 5) were installed. Each station consists of two adjacent streams (~10 m apart), of which one is polluted and the other one is clean. As reflected by the soil survey, the downstream station consists of more fine-grained soil, rich with gypsum layers while the upstream section consists of more homogeneous coarse-alluvium.

The trenches of the downstream station are in NE-SW orientation, perpendicular to the streams (that head to the SE). The cross section of the polluted stream is about 3.1 m wide and its depth is 0.15 m. The stream is not symmetrical; its lowest part is located near the eastern bank of the stream (Figure 6a). The cross section of the clean stream is about 3.0 m wide and its depth is 0.10 m deep. This stream is fairly symmetrical (Figure 4a).

The sediment sequence of the polluted stream is composed of 4 pedo-sedimentary units which contains sand and gypsum. The sequence is described in Table 1. The depth of the visually polluted soil ranges between 10 cm in the western edge of the stream till 26 cm at the deepest point of the stream.

The sediment sequence of the clean stream is very similar to the one of polluted stream. It is composed of 5 pedo-sedimentary units as described in Table 2.

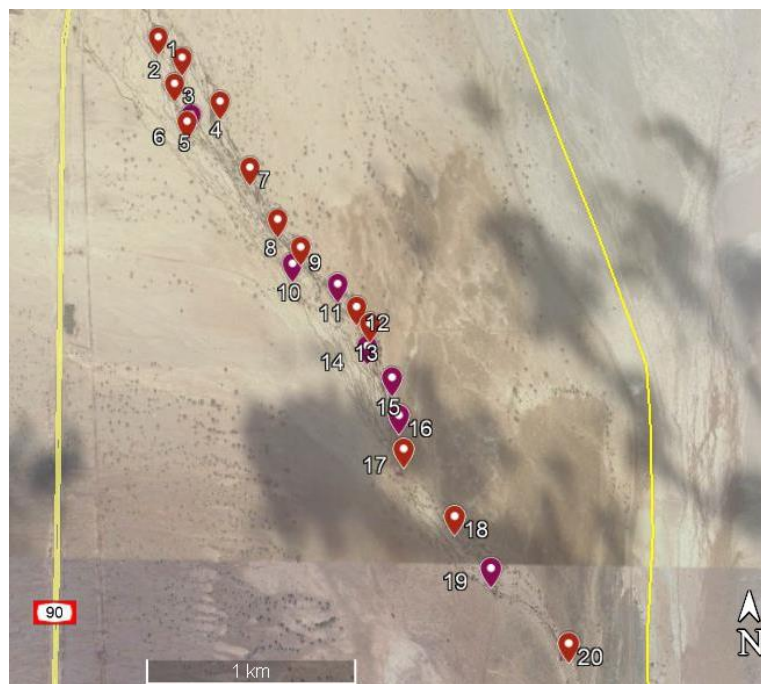


Figure 4: Location map of the points sampled for soil texture. In each point sampling was conducted at several depths, from the soil surface up to 1 m depth. The oil-polluted streams can be seen on the background.



Figure 5: Location map of the monitoring stations (blue color) and of the points from which soil was sampled for incubation experiments (Bordeaux color). In the inset: a close up view of the downstream monitoring station

Table 1: The sediment sequence in the polluted stream of the downstream section

Unit	Depth (cm)	Thickness (cm)	Description
I	0 – (25-50)	25-50	Alternating layers of well bedded sand with medium gravel (0.5-1.5 cm). Sharp and wavy boundary.
II	(25-50) – (85-95)	35-70	Alternating layers of hard and continuous gypsum (1-2 cm thick) with single grain sand (2-5 cm thick). The lower part consists of thicker (5-7 cm) gypsum layer. Distinct and wavy boundary.
III	(85-95) – (110-140)	15-55	Massive sand, common (25%) gypsum nodules 2-3 mm. Distinct and wavy boundary.
IV	(110-140) - 160	+ 20	Massive sand.

Table 2: The sediment sequence in the clean stream of the downstream section

Unit	Depth (cm)	Thickness (cm)	Description
I	0 – (30-40)	30-40	Alternating layers of well bedded sand with medium gravel (0.5-1.0 cm). Sharp and wavy boundary.
II	(30-40) – 80	40-50	Alternating layers of hard and continuous gypsum (1-2 cm thick) with single grain sand (2-5 cm thick). Distinct and smooth boundary.
III	80 – 115	35	Hard and continuous gypsum.
IV	115 - 165	50	Massive sand, common (25%) gypsum nodules 2-3 mm. Distinct and smooth boundary.
V	165 - 180	+ 15	Massive sand.

The upstream station is located a few tens of meters from the Arava highway about 2 km from the downstream station (Figure 5). The sections are in NE-SW orientation (30°), perpendicular to the streams (that head to the SE), and are 8 m apart from each other. The stream cross section of the polluted stream is about 2.0 m wide and 0.40 m deep. The section is symmetrical (Figure 7). The sediment sequence is composed of 3 pedo-sedimentary units (Table 3). The polluted sediment lies in depth of about 17 cm in the center of the stream and is covered by young sediments. The thickness of the polluted sediment is 10 cm in the middle of the stream. The oil thickness and thinning towards the stream edges.

The cross section of the clean stream and is about 2.4 m wide and 0.17 m deep; the section is of asymmetrical shape where the lowest part is located near the NE bank of the stream (Figure 3). The sediment sequence is very similar to the polluted site (Table 4).

Table 3: The sediment sequence in the polluted stream of the upstream station.

Unit		Depth (cm)	Thickness (cm)	Description
I		0 – 55	55	Alternating layers of well bedded sand with medium to large gravels (0.5 cm, 3-5 cm). Total gravels 40-50%. Gradual and smooth boundary.
II		55 – 90	35	Same as above, few (5%) gypsum nodules 2-3 mm. Gradual and smooth boundary.
III		90 – 170	+ 80	Same as above, more gravels (50-60%), few (10-15%) gypsum nodules 2-5 mm. Gypsum also partially coat gravels.

Table 4: The sediment sequence in the clean stream of the upstream section.

Unit		Depth (cm)	Thickness (cm)	Description
I		0 – 50	50	Alternating layers of well bedded sand with medium to large gravels (0.5 cm, 3-5 cm). Total gravels 40-50%. Gradual and smooth boundary.
II		50 – 85	35	Same as above, few (5%) gypsum nodules 2-3 mm. Gradual and smooth boundary.
III		85 – 150	+ 65	Same as above, more gravels (50-60%), few (10-15%) gypsum nodules 2-5 mm. Gypsum also partially coat gravels.

In both stations and in each stream, sensors were installed on the walls of the 1.8 m depth trenches that were dug for this purpose and closed immediately after installation. The scheme of the sensors in the section is presented in Figures 6 and 7 for the downstream and upstream, respectively. Twelve sensors were installed in each trench; 9 TDT sensors (Acclima, USA) and 3 TDR sensors (True TDR, Acclima, USA). The shallowest sensors were installed at a depth of 7 cm below the soil surface while the deepest sensor was installed in 120 cm depth. As the area is extremely arid, it is expected that infiltration depth will be rather shallow, specifically in the water repellent soils. Therefore, 4 sensors were installed in 7 cm depth, 3 in 15 cm depth, 2 in 30 and in 60 cm depth and one sensor in 120 cm depth. The TDR and TDT sensors measure the soil volumetric water content, the temperature and the bulk electrical conductivity which serves as an indicator of solute transport. TDR measurement is considered a little more accurate than TDT measurement. Also, the TDR probe is more easily inserted into the soil without causing the disturbance typical of TDT installation. Additionally an automatic rain gauge (TB, Young, USA) was installed above surface to record rain amount and intensity. Measurements are carried out automatically every 15 minutes. The data is stored in a data logger (CR300, Campbell Scientific, USA) and transmitted daily to the server of the Geological Survey. Data collection was programmed such that when rain starts, measurements are performed at 2 minutes intervals until 24 hours after the rain event. After that, measurements resume to 15 minutes intervals.

To better characterize the sections, undisturbed soil samples were taken to calculate porosity, bulk density, water content and soil texture (Figures 6,7). Sampling was conducted using a dedicated kit for undisturbed soil sampling. (Eijkelkamp, the Netherlands). Samples were weighted in the lab within 2 days from collection to maintain their field water content. Additional soil samples were taken from the polluted section to measure soil hydrophobicity and hydrocarbon content.

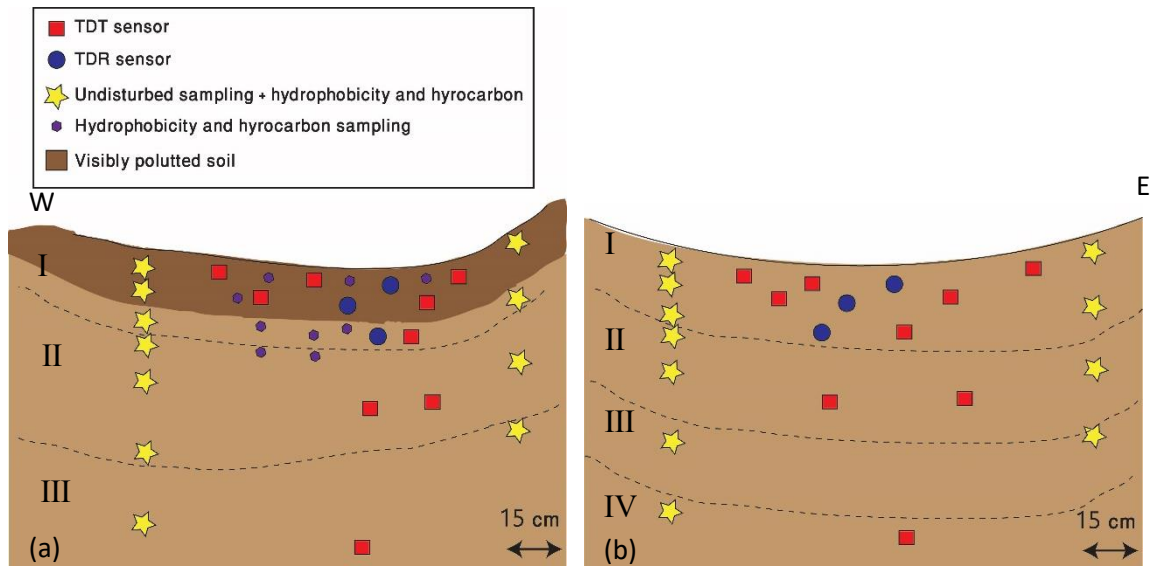


Figure 6: Schematic layout of the sensors and sampling locations in the (a) polluted trench (b) clean trench of the downstream station. Sensors are installed at depths of 7, 15, 30, 60 and 120 cm below surface. Dashed lines represent the boundaries between sedimentary units and roman numbers correspond to the units in Tables 1 and 2. Note that the deepest unit is not shown in the figure.

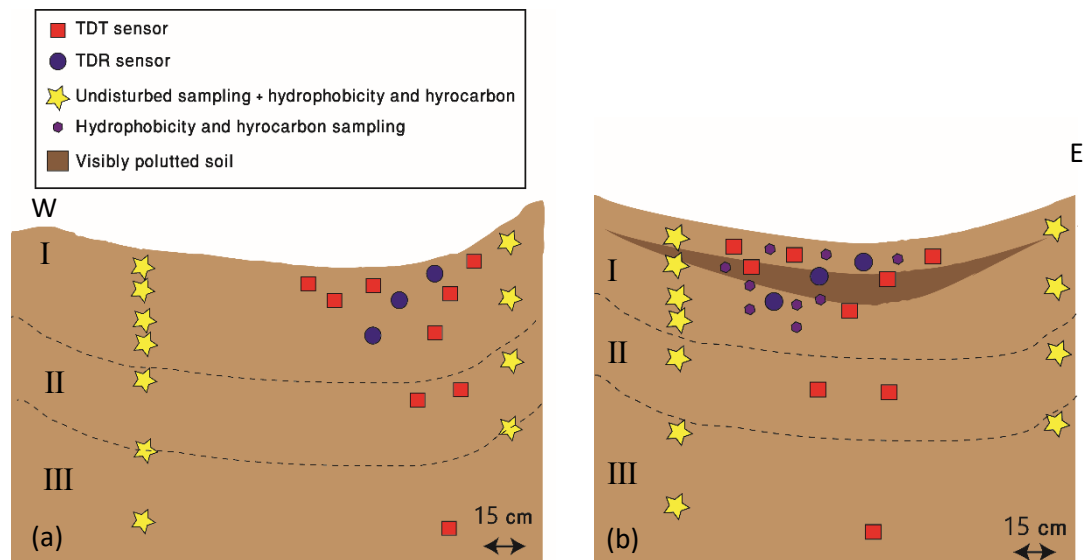


Figure 7: Schematic layout of the sensors and sampling locations in the upstream station at the (a) clean trench and the (b) polluted trench of the upstream station. Sensors are installed at depths of 7, 15, 30, 60 and 120 cm below surface. Dashed lines represent the boundaries between sedimentary units and roman numbers correspond to the units in Tables 4 and 3, respectively. Note that the deepest unit is not shown in the figure.

Incubation experiments

Soil batches (of about 2 kg each) taken from the 1975 and 2014 spill sites (Figure 2) in late May 2017 were incubated in different conditions to assess the persistence of soil hydrophobicity and oil attenuation. The experiment matrix included 10 treatments; 7 treatments for the 2014 polluted soil and 3 treatments for the 1975 soil. The 2014 polluted soil was incubated in 20% and 50% water saturation (calculated based on a bulk density of 1.5 g/cm^3), in 20% saturation with the addition of nutrients, 20% saturation with the addition of surfactant, and in 20% saturation with addition of both nutrients and surfactant. Air-dry polluted soil was used as a control and clean soil from a nearby stream was used for comparison. The 1975 polluted soil was incubated in 20% saturation, 20% saturation with the addition of nutrients and air-dry soil was used as a control.

Nutrient solution was prepared by dissolving 3 g/l ammonium sulfate and 150 mg/l potassium phosphate in tap water (deionized water was not used to avoid soil dispersion). In this experiment, 200 mg/l of Rhamnolipids (Agaetech, USA) were used to test the effect of surfactant on oil degradation and hydrophobicity reclamation. Rhamnolipids were chosen since they are model biosurfactants which were extensively investigated in the context of bioremediation (Mulligan et al., 2005).

Incubated soils were stored in containers at room temperature. Soil was stirred every two days to aerate the soil and homogenize the sample. Once a week, the soils were weighted and deionized water was added to make up for evaporative loss. Soil batches were sampled periodically (once a month in the beginning up to every 3 months, at the end of the incubation) to measure the level of hydrophobicity and hydrocarbon content.

Hydrophobicity characterization

The degree of soil hydrophobicity and water repellency was evaluated by the water drop penetration time (WDPT; Letey, 1969), molarity of ethanol droplet (MED; Watson and Letey, 1970) techniques and by measuring the drop contact angle (DCA; Bachmann et al., 2000) between the water and the soil.

Soil was dried in 65°C oven for two days and left in open air for another day. Soil was then sieved to 2 mm and placed on a petri dish generating a surface as smooth as possible (Takawira et al., 2014). In the WDPT method, the degree of hydrophobicity was assessed by measuring the time for a drop of water ($50\mu\text{L}$) to penetrate the soil surface. The results are classified according to the hydrophobicity groups of Dekker and Lungerius (1990): group 1-wettable ($\text{WDPT} < 5\text{s}$); group 2-slightly wettable ($5\text{-}60\text{ s}$); group 3-strongly non-wettable ($60\text{-}600\text{ s}$); group 4-severely non-wettable ($600\text{-}3600\text{ s}$); group 5-extremely non-wettable ($>3600\text{ s}$). In the MED test, the hydrophobicity was assessed by pipetting a $50\mu\text{L}$ droplet of ethanol solution on the soil surface and finding the lowest molarity of ethanol solution which penetrates the soil surface within 5 s.

DCA measurement was conducted by the sessile drop contact angle method (Bachmann et al., 2000). Briefly, air dry sieved soil particles (<0.5mm) were spread on a smooth microscope glass slide wrapped with double-sided tape. The surplus grains were removed by knocking the slides carefully. Then, five 10 µl drops of double-distilled water were placed on the surface of the soil layer by a microsyringe. Records of the water drops were taken by a microscope connected to the computer (OCA20, Dataphysics, Germany). The contact angles were then calculated using the SCA software (Dataphysics, Germany). .

Hydrocarbons extraction and analysis

Ten grams of the soil from the incubation experiments were dried in the oven at 65°C for 2 days. Five grams of the dried soil were placed into 40 ml glass vial and mixed with 1 gram of anhydrous Na₂SO₄, followed by addition of 15 ml of dichloromethane. The vials were sonicated at room temperature for 10 min. The extract was filtered and analyzed by GC-MS.

Hydrocarbons were analyzed with GC-MS (6890N-5975; Agilent) equipped with a Rxi-5HT capillary column (30m, 0.25 mm, 0.25µm; Restek). GC oven was heated from 50°C to 300°C at 10°C/min and held at this temperature for 10 min. Helium was used as a carrier gas. Injector was held at 280°C. Individual n-alkanes were identified based on the retention time of the standards (nC₁₀₋₄₀; Sigma-Aldrich). Areas of GC-MS peaks were used calculation of indicative ratios.

Total Petroleum Hydrocarbon (TPH) analyses were performed at a certified lab (Aminolab, Israel) according to EPA 8015 standard method.

Measurements of unsaturated hydraulic properties

Soil water retention curves and hydraulic conductivity functions were measured using the Hyprop system (UMS GmbH, Munich). The system is based on the frequently used evaporation method proposed by Wind (1968) and further developed (Schindler et al., 2010).

Three hundred and seventy-five grams soil were oven-dried in 65 °C for two days and sieved to 2 mm. Then, the soils were well-mixed with double distilled water (DDW), packed into 8 cm, 250 cm³ cylinders at a bulk density of 1.5 g/cm³ and put into a container. DDW was poured into the container until the water level reached the cylinder height to fully saturate the soils samples by capillary rise. Full saturation was obtained after about a week with the saturated water contents being around 0.35~0.4.

Next, the cylinders with the soil samples were connected to the Hyprop system and left to evaporate. During evaporation, the soil samples were weighed at least once a day and the mass of the measurement units was recorded. As shown in Fig. 8, two tensiometers were placed inside the soil sample at 3.75 cm and 1.25 cm from the bottom to measure the matric heads.

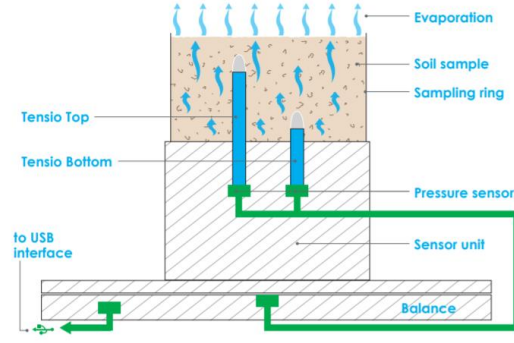


Figure 8: Schematics of the Hyprop system (UMS GmbH, 2015)

The average volumetric water content $\bar{\theta}$ [L^3/L^3] was calculated using the weight and matric head according to:

$$\bar{\theta}(\bar{h}) = \frac{M_{(t)} - M_s}{V \cdot \rho_w} \quad (1)$$

where $\bar{h}$ is the mean matric head [L] of the reading of the two tensiometers at time t , $M_{(t)}$ is soil sample mass [M] at time t , M_s is the mass of the oven-dried soil at 105 °C, V is the sample volume [L^3], 250 ml and ρ_w is the water density [M/L^3], 1 g/cm³. The WRC is then obtained from $\bar{h}$ and $\bar{\theta}$.

The evaporation flux q [L/T] through the soil sample was calculated by using two consecutive weights according to:

$$q = \frac{M_t - M_{t+1}}{A \Delta t \cdot \rho_w} \quad (2)$$

where A [L^2] is the cross sectional area of the cylinder.

The unsaturated hydraulic conductivity K [L/T] was then calculated as follows,

$$K(\bar{h}) = -\frac{q/2}{\Delta z / \Delta H} \quad (3)$$

where H is the hydraulic head [L], which is the sum of the matric head and elevation, and Δz is the height difference between the tensiometers equal to 2.5 cm.

At the dry range of the water retention curve, measurements were obtained by the dew point method using the WP4C device (Decagon Devices Inc., USA). The WP4C PotentiaMeter system is based on equilibrium of potential between water in a soil sample and the relative humidity of the air space above the sample (Dane et al., 2002). Relative humidity is calculated using the dew point temperature of moist air which is measured by a chilled mirror, and the soil sample temperature which is measured by an infrared thermometer. The soil water potential is calculated using the Kelvin equation (Dane et al., 2002). The potential found is the sum of the matric and osmotic potential. This dew point method was applied to get the wetting and drying retention curves. For the drying curves, sieved (<2mm) oven-dried soil samples were saturated and kept overnight to stabilize. Each soil sample was exposed to evaporation, put into the WP4C chamber to measure the total potential and was weighted to assess the loss of water. This process was repeated until the water content reached to 0.5%. For the wetting curves, a specific amount of water was added to the sieved (<2mm) oven-dried soil

samples to reach a specific water content and kept overnight. Then the water potential and real water content were measured.

The WP4C method is adequate to measure the matric head of samples with matric head values greater (in absolute value) than 50 meter H₂O (Decagon-Devices, 2013). Therefore, it complements the Hyprop device in measuring the WRC at the high-suction range, which the Hyprop is not suited for.

Measured data of the water retention curve were fitted to the Van Genuchten's relation (van Genuchten, 1980),

$$\frac{\theta - \theta_r}{\theta_s - \theta_r} = [1 + (\alpha|h|)^n]^{-m} \quad (4)$$

where θ is the water content, θ_r is the residual water content, θ_s is the porosity, h is the matric head, α , n and m are empiric parameters where commonly $m = 1 - 1/n$. The parameter α is related to the inverse of the air-entry value, and n is a measure of the pore-size distribution.

For the Hyprop results the parameters n and m were independently fitted (i.e., not keeping the dependence between m and n) to allow more flexibility while in the WP4C data the dependence was kept ($m=1-1/n$). The WP4C data were fitted by using the RETC code (Soil Salinity Lab, USA). The Hyprop evaporation data combined with the WP4C data were fitted by using Hypropfit software (UMS, Germany).

Hydraulic conductivity obtained from the Hyprop experiment was fitted to the Maulem model (Maulem, 1976) combined with the van Genuchten's relation (van Genuchten, 1980),

$$K(h) = K_s(1 + (\alpha|h|)^n)^{-\tau(\frac{1}{n}-1)} \left[1 - (\alpha|h|)^{n-1} [1 + (\alpha|h|)^n]^{\frac{1}{n}-1} \right]^2 \quad (5)$$

where $\tau[-]$ is the tortuosity parameter, often set to 0.5. In our case, $\tau[-]$ was fitted to increase the flexibility of the fitting.

Quantification of microbial oil degraders

Soil DNA extractions and quantitative PCR (qPCR)

Microbial DNA from 0.25 g dry weight (dw) soils was extracted by a soil DNA extraction kit (DNeasy PowerLyzer PowerSoil Kit, Mo Bio, USA) in the following treatments: 1975 treatments (1975 control July 2017; 1975 control Jan 2019; 1975 20% Jan 2019; 1975 20% Nut Jan 2019) and the 2014 treatments (2014 control Jul 2017; 2014 control Jan 2019; 2014 20% Jan 2019; 2014 50% Jan 2019; 2014 20% Nut Jan 2019; 2014 20% Surf Jan 2019; 2014 20% Nut Surf Jan 2019; 2014 clean 2014 Jul 2017; 2014 clean Jan 2019). The DNA extraction was conducted in duplicates according to the manufacturer instruction. The extracts were kept for the quantification of genes *alkB*, *nahAc*, and *dmpN*. The sequences of degenerated primer sets used in this study are presented in Table 5. The reaction mixture (20 μ l) contains 10 μ l enzyme mix (qPCRBIO SyGreen Blue Mix Lo-Rox, PCR Biosystems Inc, USA), 0.4 μ M forward primer, 0.4 μ M reverse primer, 1 μ l DNA template and 8.2 μ l nuclease-free water. Real-time PCR was conducted by a CFX96 Touch™ Real-Time PCR Detection Systems (Bio-Rad, USA). The amplification cycle was: initial denaturing at 94 °C for 30s, followed by 40 cycles of denaturing at 94 °C for 5 s, annealing at 64 °C for 15 s,

extension at 72 °C for 10 s; and then hold at 55 °C for 30 s. qPCR amplification products were run using 1.5 % agarose gel electrophoresis to check for primer dimers and non-specific bands.

Table 5: Primer sequence for qPCR

Primer name	Target gene	Sequence	Reference
Alk-BFB	alkane monooxygenase (<i>alkB</i>)	5'-GGT ACG GSC AYT TCT ACR TCG A-3'	Marchant et al., 2006
Alk-BRB		5'-CGG RTT CGC GTG RTG RT-3'	
NAH-F	Naphthalene dioxygenase (<i>nahAc</i>)	5'-CAAAARCACCTGATTYATGG-3'	Baldwin et al., 2003
NAH-R		5'-AYRCGRGSGACTTCTTTCAA-3'	
PHE-F	Phenol hydroxylase (<i>dmpN</i>)	5'-GTGCTGACSAAYCTGYTGTT-3'	
PHE-R		5'-CGCCAGAACCAYTTRTC-3'	

The numbers of copies of the *alkB*, *nahAc*, and *dmpN* genes were quantified relative to a standard curve of serial dilution of stocks used as a positive control. Duplicates of the standard curves were prepared by serial dilution (10^{-1} to 10^{-7}) of the plasmid. Clean PCR products of *alkB* were used for the cloning and transformation according to the protocols of CloneJET PCR cloning (Thermo Scientific, USA).

Number of copies of genes for the plasmids used for standard curves were calculated according to Ritalahti et al. (2006),

$$N = \frac{c \times A \times v}{L \times 10^9 \times 660} \quad (7)$$

Where N is number of copies [copies/ μ l], c is the DNA concentration [ng/ μ l], A is the Avogadro's number, equal to 6.02×10^{23} , v is the volume of template [μ l], and L is the amplicon length [base pairs, bp].

The number of copies of target genes per reaction mix were determined from the standard curve based on the cycle number at the set fluorescent intensity. The number of copies of target genes per gram dw soil was calculated as,

$$n = \frac{N \times V}{w} \quad (8)$$

where N is the number of copies per volume [copies/ μ l], n is the number of copies per gram dry weight soil [copies/g], V is the volume of extracted DNA, w is the weight of soil sample used in the DNA extraction.

Most probable number

The method is a modified version of the 96-well microplate most-probable-number (MPN) method (Haines et al., 1996). Briefly, bacterial cells were extracted from the soil by shaking 5 g soil with 45 ml sterile Bushnell-Haas (BH) broth (Sigma, USA) for

30 min. The supernatant was serially diluted in sterile BH medium. Six 400 µl replicates of each dilution were added to 48-well microplates. Either hexadecane (4 µl) or naphthalene (30 µl, 1% dissolved in silicone oil) was used as a substrate for the enumeration of total alkanes and aromatic degraders, respectively. Two microplates with BH medium without hexadecane and with soil dilution alone were used as negative controls for total alkane and aromatic degraders, respectively. The microplates were incubated at 25 °C for four weeks in a humid box. Growth-positive wells were identified by the addition of the tetrazolium compound iodonitrotetrazolium violet (INT). The colorless tetrazolium compound replaces the oxygen as the terminal electron acceptor during the respiration of active cells (Smith and Mcfeters, 1997) and turns into purple formazan. The MPN numbers were calculated according to MPN calculation program (Jarvis et al., 2010).

Isolation and evaluation of biosurfactant producers

Enrichment and isolation of biosurfactant producing strains

To isolate the biosurfactant producing microorganism, five grams of contaminated soil were inoculated into 100 ml Bushnell HassHaas Broth (BH) mineral medium containing hexadecane (1% v/v) as a carbon source and incubated at 25 °C for 7 days. Then, 50 µl of the culture broth was transferred into a fresh BH medium and incubated under the same conditions. This procedure was repeated 3 times to en-rich the microbial population. After this, aliquots were spread on BH agar plates with hexadecane (1% v/v) used as the carbon source. These plates were incubated at 25 °C for 7 days. Several colonies that appeared on the agar plates were select-ed for further purification. These colonies were inoculated into fresh sterile BH agar plates and inoculated into Trypticase soy broth (TSB) agar plates to check their purification. 12 single colonies were selected randomly and inoculated into fresh BH mineral medium and incubated on a shaker at 25 °C for one week.

Screening of biosurfactant production

Bacteria were cultivated in a 250 ml flask with 50 ml of BH mineral medium with hexadecane (1% v/v) (Sigma, USA). A loop-full of a single colony grown on the BH agar plates with hexadecane (1% v/v) was inoculated in the flasks of sterilized BH medium and incubated in a shaker at 200 rpm and 30°C for one week. At the end of incubation, the cells in the culture broth were removed by centrifuge (Heraeus, Germany) at 6000 rpm and 4 °C for 15 min and then filtered through 0.45 µm filters (Merck Millipore Ltd., Ireland). The cell-free culture solutions were used for the following screening tests: oil spreading assay, emulsification index (E24) assay, and surface tension assay .

The oil spreading experiment is a modified version of the method described by Morkikawa et al. (2000). Briefly, 10 µl crude oil, which was obtained from Tzuk Tamrur 3 borehole near Arad, was added to the surface of 20 µl distilled water in a petri

dish, followed by the addition of 10 µl cell-free culture broth. The diameter of the oil-free clear zone is a measure of the oil displacement activity. A result was considered positive for biosurfactant production when the diameter of the clear zone was at least 5 mm (Thavasi et al., 2013). As the crude oil we used was too dense and the spreading activity was affected by many factors such as the temperature and the time that the oil was in contact with the water surface, the results of the oil spreading assay were not comparable between replicates. Therefore, the oil spreading assay was used as a qualitative method to justify the biosurfactant production activity.

The measurement of emulsification activity is described in Cooper and Goldenberg (1987). Two ml hexadecane and two ml cell-free culture broth were vortexed at the maximal speed for 2 min and then kept static for 24 h. Emulsification was then characterized by the E24 index which is the ratio of the height of the emulsion layer to the total height of the fluid.

The surface tension assay is one of the common methods to screen the biosurfactant production (Sidkey et al., 2016). The surface tension measurement of the cell-free broth mentioned above was conducted by the du Nouy ring method (McInerney et al., 1990) in a K6 tensiometer (Kruss GmbH, Hamburg). The surface tension measurement of the cell-free broth mentioned above was conducted by the Wilhelmy plate method in a tensiometer (DCAT, Dataphysics Co. Germany). For the measurement of the surface tension, 15 ml of the culture were equilibrated in a small weighing dish. Measurements were conducted in triplicates.

Another mineral salt medium in which glycerol serves as the carbon source was prepared as followed (per liter): 20 g glycerol, 0.7 g KH₂PO₄, 0.9 g Na₂HPO₄, 2 g NaNO₃, 0.4 g MgSO₄ · H₂O, 0.1 g CaCl₂ · 2H₂O, and 2 ml of a trace solution containing 2 g FeSO₄ · 7H₂O, 1.5 g MnSO₄ · H₂O, and 0.6 g (NH₄)₆Mo₇O₂₄ · 4H₂O. Isolates were cultured in the above mineral medium at 30 °C for 3 days. The same procedure as mentioned above was conducted to get the cell-free culture broth and then test the surface tension. The goal of this measurement was to compare the production of biosurfactants when hexadecane is used as a carbon source to their production when glycerol is used instead.

DNA extraction and identification by 16S rRNA sequencing

The isolation of pure genomic DNA from the isolated strains was extracted by a commercial kit (GenElute Bacterial Genomic DNA kit, Sigma-Aldrich, USA) according to the manufacturer's instruction. The concentration of eluted DNA was measured by NanoDrop Spectrophotometer (ND-1000, Thermo Fisher Scientific, USA).

The primer set 341f (5'- CCTACGGGAGGCAGCAG-3') (Muyzer et al., 1993) and 907r (5'- CCGTCAATTCMTTGTGAGTTT) (Lane, 1991) was used to amplify the 16S rDNA of the isolates. The volume of the 16S rDNA PCR reaction mixture was 50 µl, consisting of 25 µl enzyme mix, 2 µM forward primer, 2 µM reverse primer, 2 µl DNA template and 19 µl DNA-free water. The procedure was as followed: initial

denaturing at 95 °C for 5 min, 35 cycles consisting of denaturing at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 20 s and final extension hold at 72°C for 5 min. After the examination of the size of amplified PCR products on 1.5 % agarose gel, the sequencing was performed on Applied Biosystems 3500 xL Genetic Analyzer (Applied Biosystems) at the Genomic unit of Ben Gurion University of the Negev. The 16S rRNA gene sequences of the isolates were aligned and compared with known reference 16S rRNA gene sequences in Genbank database using BLAST search at the National Center for Biotechnology Information. The phylogenetic tree was constructed with Geneious Prime (New Zealand) using a neighbor-joining method.

Statistical analysis

Statistical analyses were conducted by Prism 7 (GraphPad, USA). Significant differences between different treatments for the analyses were conducted by one-way analysis variance (ANOVA). When the F value from the one-way ANOVA analyses was found to be significant ($P < 0.05$), the difference between the means of two sets were compared by t-test, with the regression between sets of two variables subjected.

Results and discussion

Soil survey

The fraction of coarse particles (diameter larger than 2 mm) as a function of the distance downstream from road 90 is presented in Figure 9. Generally, the fraction of coarse material decreases with the downstream distance for all depths. This decrease is especially pronounced in the first 1500 m where, for instance, the fraction of coarse material at depth 5-10 cm of decreases from over 60% at 568 m to 11% at 1980 m.

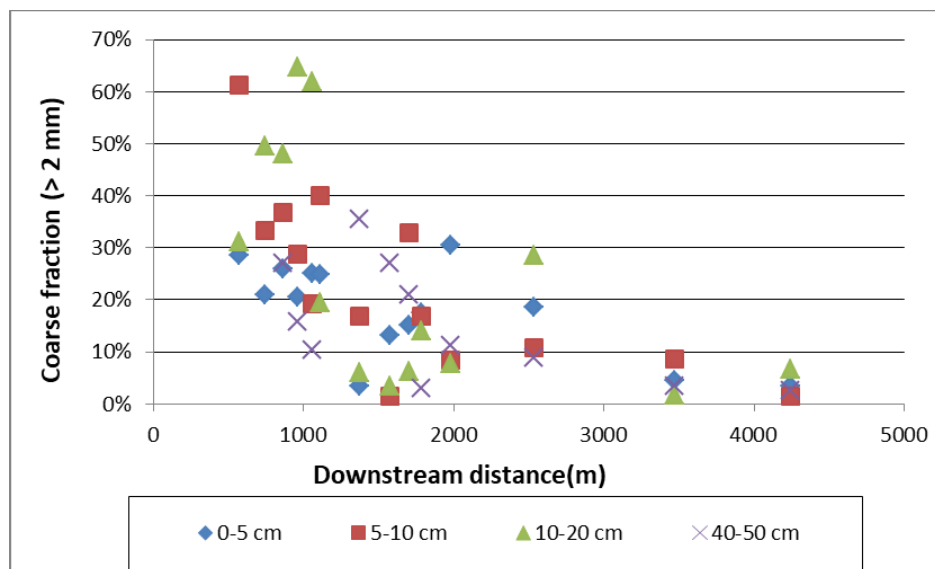


Figure 9: Fraction of coarse material as a function of the distance downstream from road 90.

The fractions of sand, silt and clay (from the total material below 2 mm) as a function of the distance downstream from road 90 are presented in Figure 10. It is shown that in most of the samples the sand fraction ranges from 60% to 80%, the silt fraction is between 10% and 30% and the clay fraction is lower than 15%. These correspond to soil textures ranging from sand through loamy sand, sandy loam, and to loam and silty loam. Most of the samples range from loamy sand to sandy loam. No spatial trend can be observed with regard to the downstream distance. The samples taken from 40-50 cm depth show higher silt and clay fractions than the samples taken from shallower depths. Given these results, it was decided to locate the monitoring station in the middle of the basin in a distance of roughly 2500 m downstream from road 90.

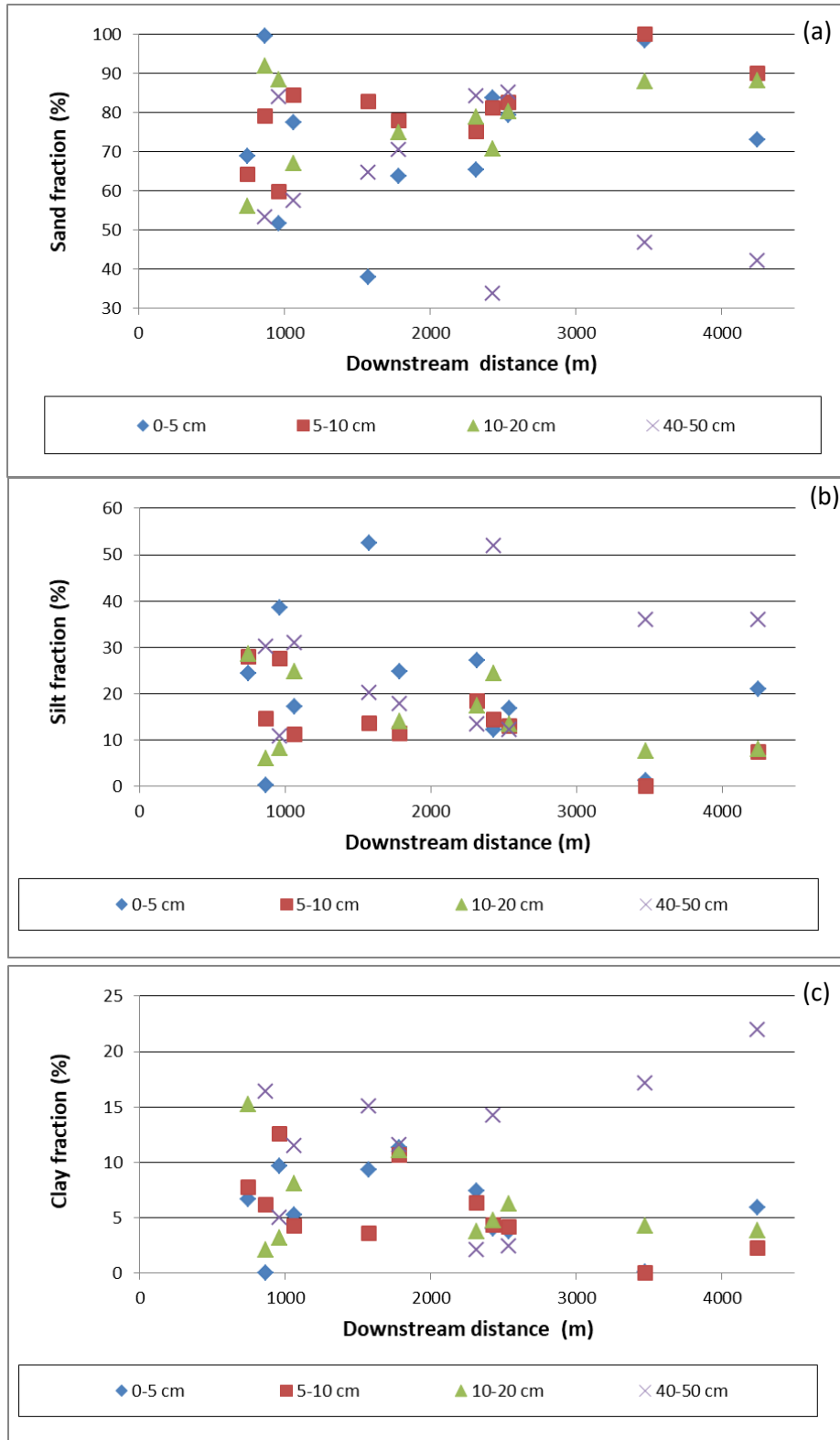


Figure 10: Fraction of (a) sand (b) silt and (c) clay from the material smaller than 2 mm as a function of the distance downstream from road 90.

Hydrological field monitoring

Bulk density, porosity and volumetric water content in the two downstream and two upstream trenches are shown in Figure 11 and 12, respectively. In each trench,

properties were measured in two vertical profiles in the stream edges from which undisturbed soil samples were taken (Figures 6 and 7). Volumetric water content was calculated by first calculating the gravimetric water content by the double weighing method and then converting it to volumetric water content using the bulk density values (Topp and Ferre, 2002).

The soil bulk density ranges between 1.4 and 1.8 g/cm³, typical values for sandy soils. In the upstream station the soil's density is a little higher in the clean trench in the upstream section, while no trend is observed in the downstream station. The average value in the upstream station ($1.62 \text{ g/cm}^3 \pm 0.09 \text{ g/cm}^3$) is higher than the one obtained for the downstream station ($1.51 \pm 0.1 \text{ g/cm}^3$). This could be attributed to the higher fraction of gravel in the upstream station. The soil's porosity ranges between 34% and 47%, which are also typical of sandy soils. Since the soil porosity is reciprocal to the bulk density this is a mirror view of the bulk density. The volumetric water content ranges from below 1% at the soil surface of the polluted stream of the downstream station to 20.5% at a depth of 120 cm at the clean stream of the downstream section. At the upstream station the variability of the water content is much lower, probably due to the more homogeneous soil section. Another reason for the high water content at the downstream station could be related to a rain event that preceded the sampling of the downstream station. The effect of the hot climate and the high evaporation rate is shown by the low water content near the soil surface.

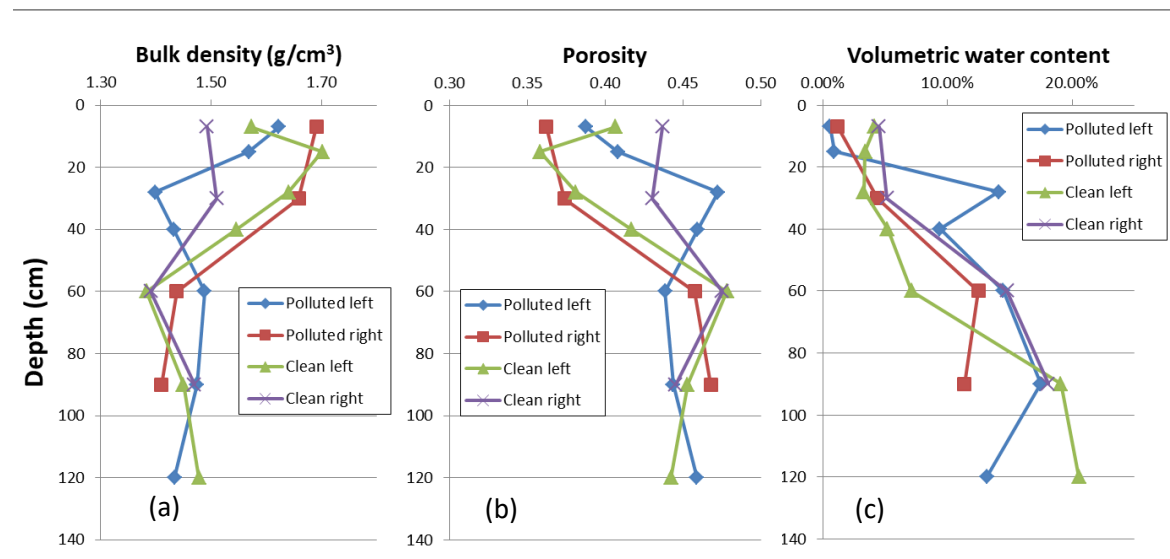


Figure 11: Profiles of (a) bulk density (b) porosity and (c) volumetric water content in the obtained by undisturbed sampling in the polluted and clean streams of the downstream station.

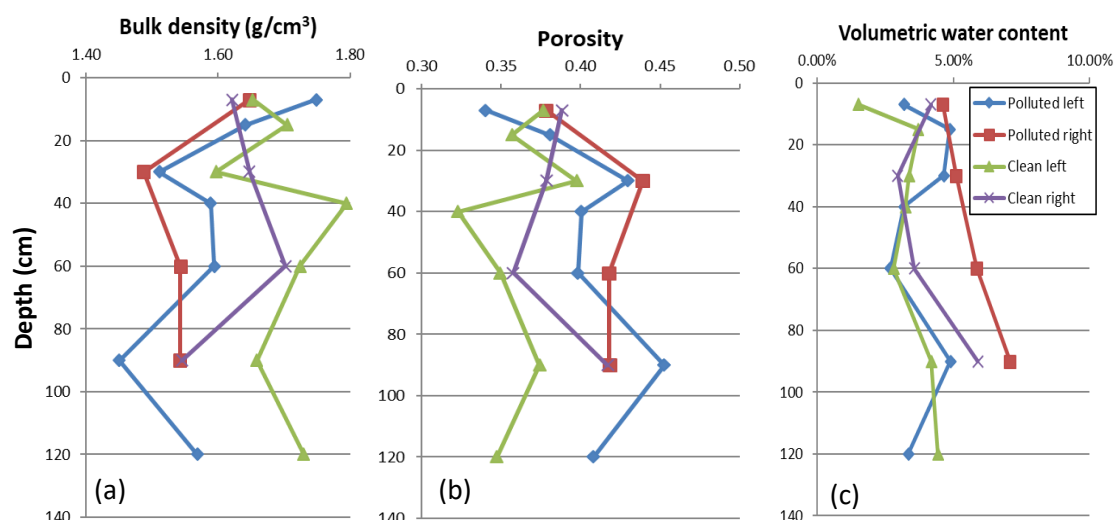


Figure 12: Profiles of (a) volumetric water content (b) bulk density and (c) porosity obtained by undisturbed sampling in the polluted and clean streams of the upstream station.

Profiles of the sand, silt and clay fractions obtained for the downstream and upstream stations are displayed in Figures 13 and 14, respectively. The soil's texture is presented on the USDA soil triangle in Figure 15.

The soil's texture of the downstream station ranges from sandy loam through loam to silt loam. The sand fraction ranges from 90% in the surface of the polluted section to 32% in the depth of the clean section. Generally, the silt fraction increases with depth from 6% on the surface to 52% at 120 cm. This corresponds to the gypsum layers that can be found in these depths. The clay fraction ranges around 13% and is relatively constant with depth (apart from a peak around 30 cm depth). In the upstream station, the soil texture ranges from loamy sand, through sandy loam, loam, sandy clay loam, silty clay loam to clay loam. Compared to the downstream station, the upstream station is richer in clay (up to ~35%) and poorer in sands (around 60% on average).

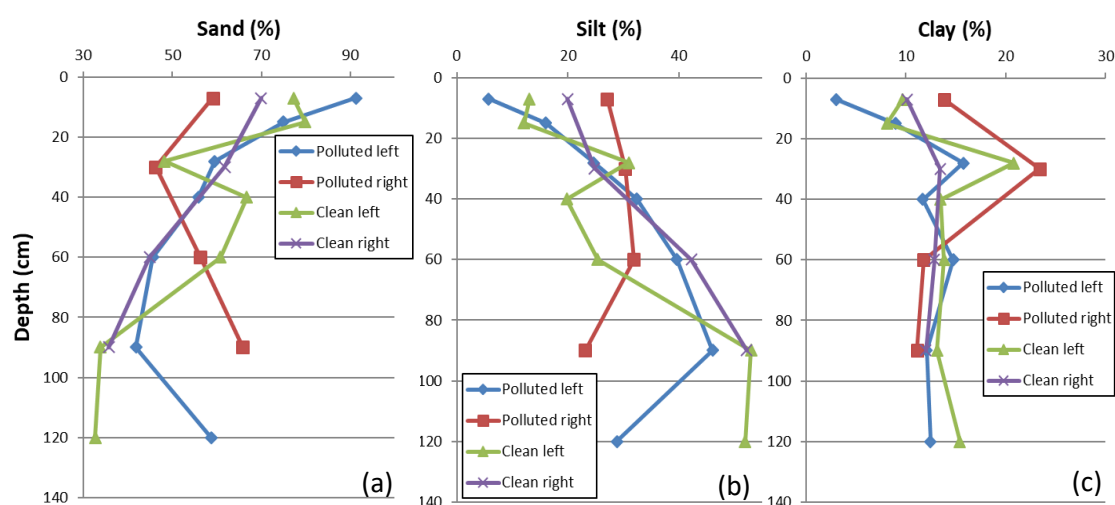


Figure 13: Profiles of (a) sand (b) silt and (c) clay fractions obtained in the polluted and clean streams of the downstream station.

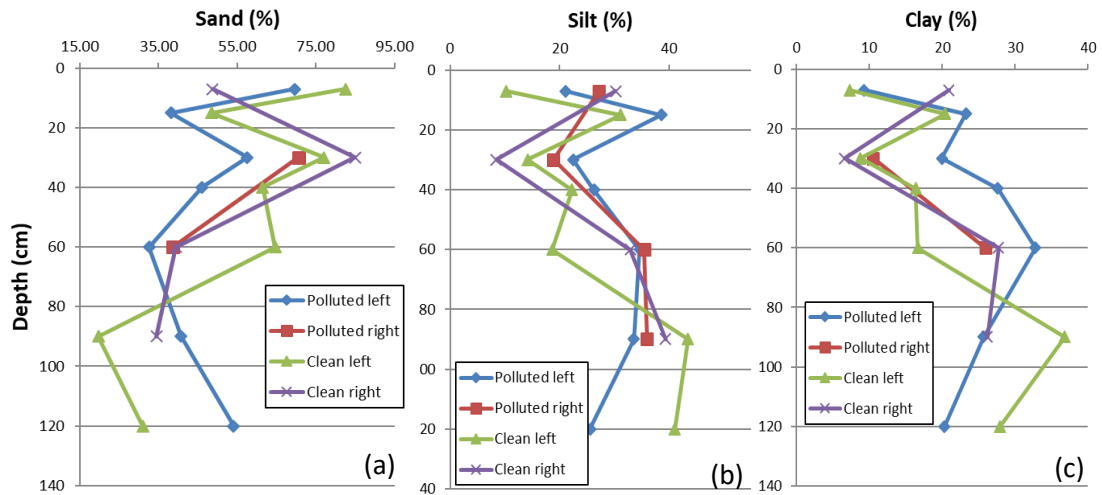


Figure 14: Profiles of (a) sand (b) silt and (c) clay fractions obtained in the polluted and clean streams of the upstream station.

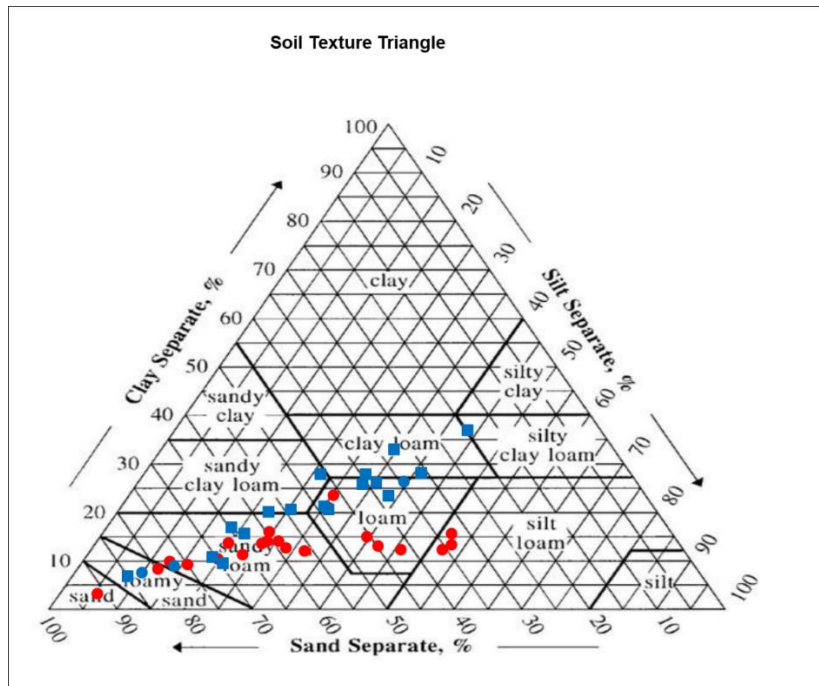


Figure 15: Soil texture of the samples from the downstream (red circles) and upstream (blue squares) stations.

While the soil texture at the two sections is similar in most parts (Fig. 15), the fraction of coarse material is much higher in the upstream station compared to the downstream station (Fig. 16).

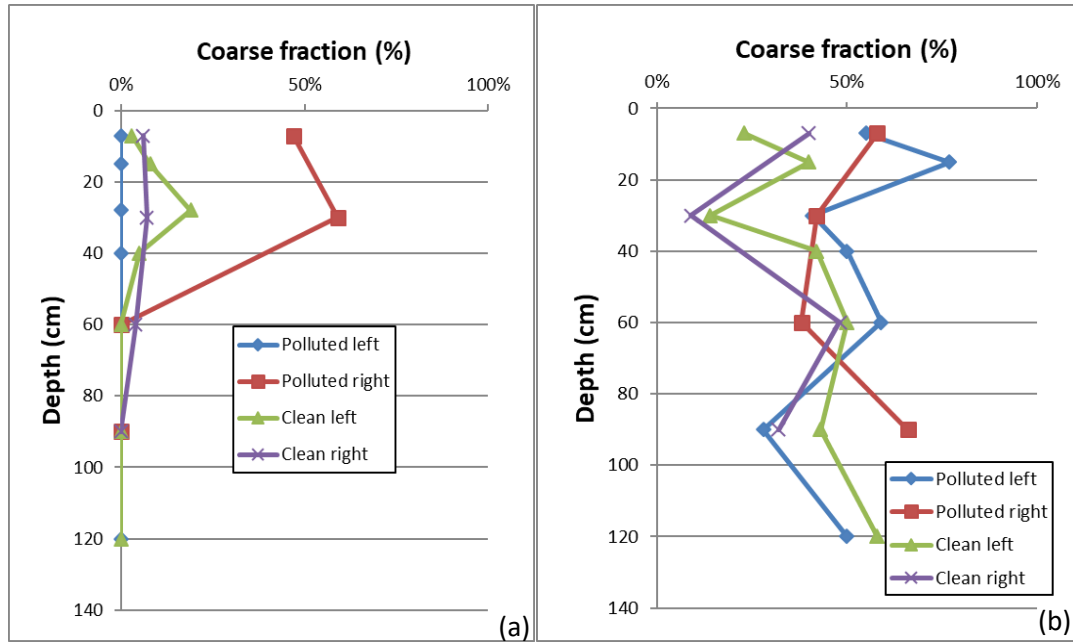


Figure 16: Fraction of coarse material (>2 mm) in the (a) downstream (b) upstream stations.

Figure 17 shows the hydrophobicity distribution in the downstream polluted section (Fig. 17 a,b) and the ratio between low to high weight alkanes (L/H; Figure 17c). An increase in that ratio represents higher abundance of low-weight alkanes common in non-degraded oil. Additionally, three representative concentrations of Total Petroleum Hydrocarbons (TPH) of samples taken from the polluted soil, 20 cm below the visually polluted soil and from the clean soil, are indicated on the figure. The top 30 cm of the profile exhibits very severe hydrophobicity, both in terms of MED and of WDPT, which reduces with the increasing depth. This corresponds with the L/H ratios and with the TPH concentrations, which exhibit high oil concentration in the polluted soil, low but detectable concentration below the visibly polluted soil and non-detectable concentration in the clean soil. In the upstream section, the polluted layer was much narrower with respect to the downstream section, with only one sample in the middle of the trench (at 10 cm depth) being hydrophobic having WDPT of 320 s, MED of 25% and characterized as strongly non-wettable.

During the two years of monitoring in the downstream station and the one-year monitoring in the upstream station, four significant rain events have occurred on the site (Figure 18a). Three of the events also induced overland flows. The first event took place on October 20 2018, shading about 10 mm of rain. The second event occurred on February 7 2019, shading 6.5 mm of rain. The third occurred on February 7 2020 with 13 mm rain and the most substantial event took place on March 13 2020 shading 26 mm, which amounts to 90% of the entire annual rainfall. Unfortunately, malfunctions in the rain gauge prevented registration of the data for some of the period and the rain data was taken from a nearby station about 2 km away from the downstream station and 3.5 km away from the upstream station. These rain events increased the water content

in the soil and can be easily observed in the measurements of the sensors of the clean downstream section, presented in Fig. 18b.

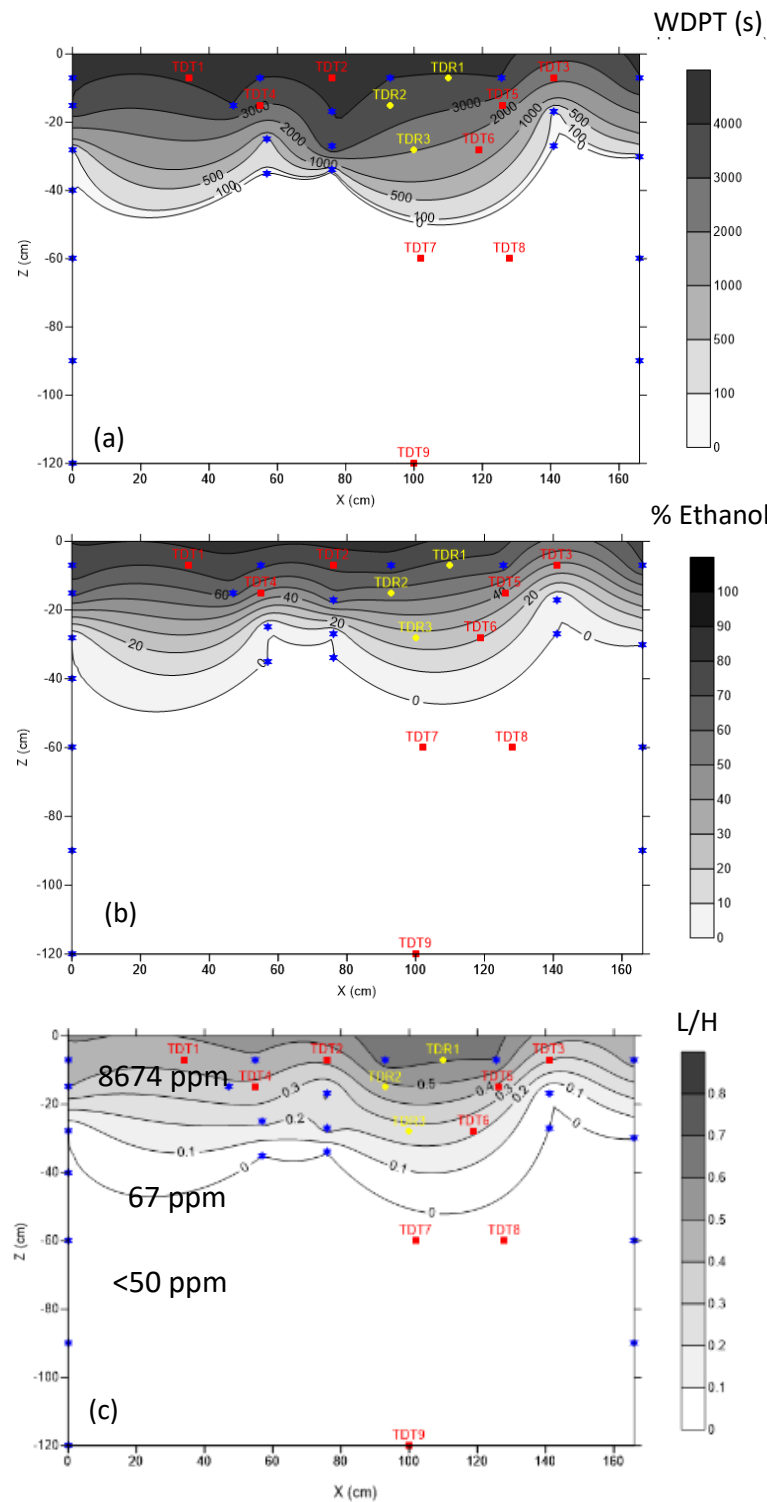


Figure 17: Hydrophobicity distribution in the polluted section of the downstream station in terms of (a) WDPT and (b) MED (c) Low- to high- weight alkane ratio. Three representative TPH concentrations are indicated in black on the contour lines. The locations of the sensors and sampling points are shown in red and yellow and in blue, respectively.

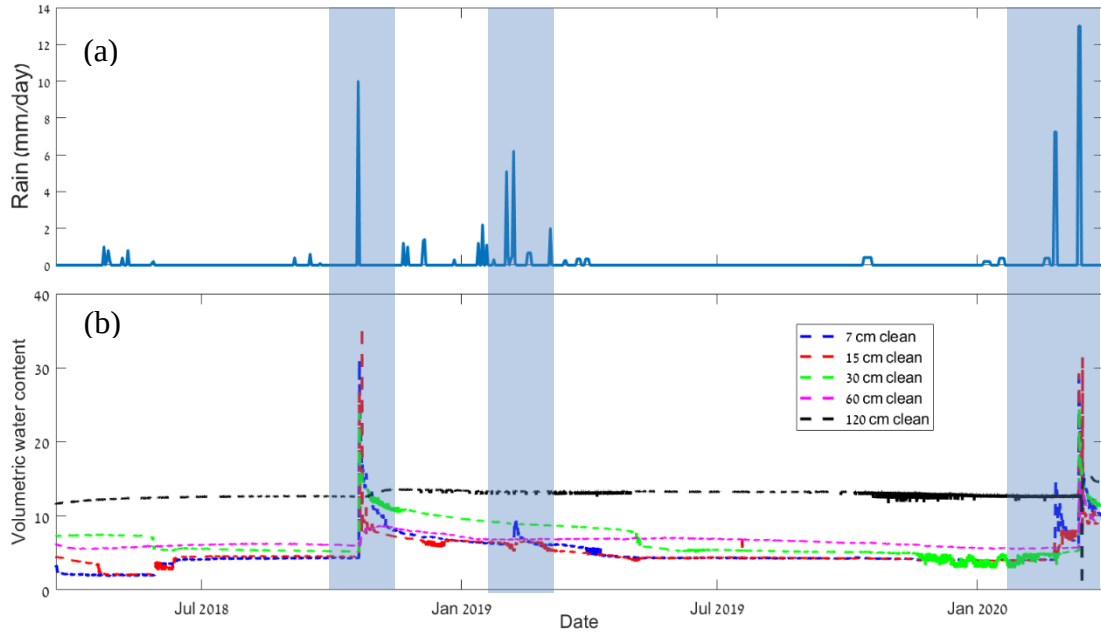


Figure 18: (a) Rain and (b) depth-averaged water content in the clean section measured in the downstream station between 20/3/2018 to 31/3/2020. Rain events, which induced overland flow and infiltration, are highlighted.

Figure 19 shows the depth-averaged water contents in the clean (Fig.19a) and contaminated (Fig. 19b) sections of the downstream section measured during and after the October rain event. In the clean section, the measured water contents show classic infiltration behavior with the shallow sensors reaching higher water contents and demonstrating earlier arrival of the wetting front. In the contaminated section, however, the wetting behavior is different. First, the water contents are much lower than in the clean sections. Second, the highest water content is obtained at depth of 15 cm, then the second highest water content is obtained at depth of 28 cm, while in a depth of 7 cm the water content is lower. This behavior might indicate preferential flow in the contaminated section.

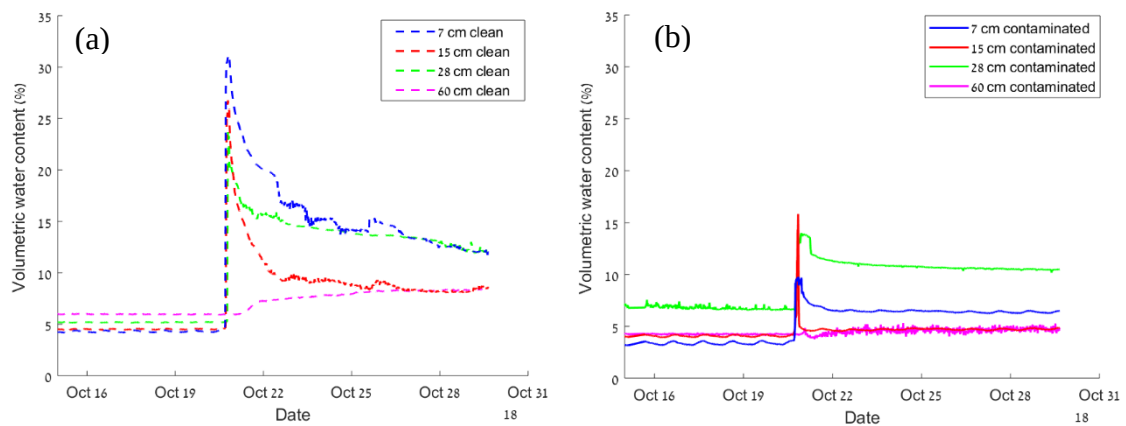


Figure 19: Depth-averaged water content in the downstream station showing the October 20 rain event. (a) Clean section (b) contaminated section.

Figure 20 shows the coefficient of variance (standard deviation divided by the average) of the water content at each depth during the rain event. It is shown that apart from the 15 cm depth, in all depths the coefficient of variance of the contaminated section is higher than that one of the clean section, strengthening the possibility that preferential flow exists in the contaminated section. Calculation of the water depth, which penetrated the soil during the rain event, yielded 43 mm in the clean section and only 11 mm in the contaminated section. This is a striking evidence of the effect of soil hydrophobicity on the infiltration capacity of the soil.

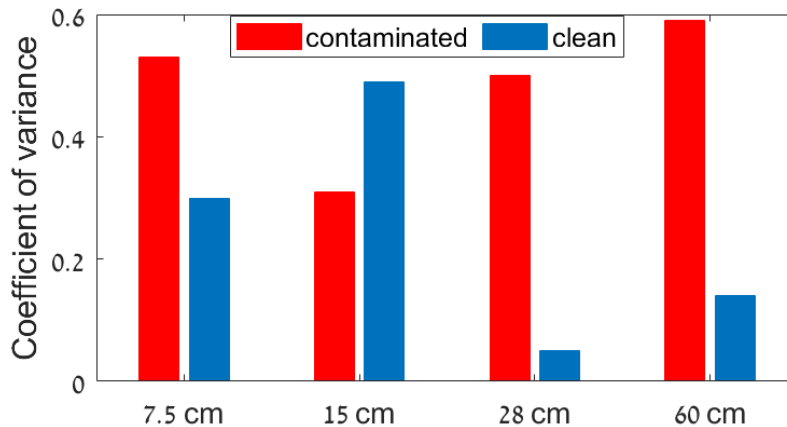


Figure 20: Coefficient of variance of the water content during Oct 2018 rain event.

Figure 21 shows the depth-averaged water contents in the clean (Fig. 21a) and contaminated (Fig. 21b) sections of the downstream station measured during and after the February 2019 rain event. As the rainfall and runoff in this event was considerably smaller than in the October rain event, the water contents are lower. In the clean section, wetting of the two shallowest depths occurs while no change in the water content is observed at a depth of 30 cm. On the other hand, in the contaminated section, just a small and short-lived increase of the 15 cm water content is shown, while no significant change is observed at a depth of 15 cm. Surprisingly, the highest water content is obtained at 30 cm depth. This is another evidence of preferential flow at the contaminated section. The increase shown in a depth of 30 cm may be related to the gypsum layer at this depth which is less permeable than the overlying soil. This may cause lateral flow of water which is detected in the two sensors at this depth. Figure 22 shows the coefficient of variance during the rain event. Similarly to Figure 20, it is shown that for most depths, the coefficient of variance for the contaminated section is higher, suggesting a preferential flow in the contaminated stream.

Figure 23 shows the depth-averaged water contents in the clean and contaminated sections of the downstream station measured during and after the February 2020 rain event. Although a substantial amount of rain fell in this event (13 mm during 2 days), since the soil was completely dry beforehand, this event did not induce overland flow and the increase in water content in the downstream station was as a result of local

infiltration alone. In the upstream station, only the contaminated stream flowed and therefore we could not compare between the two streams.

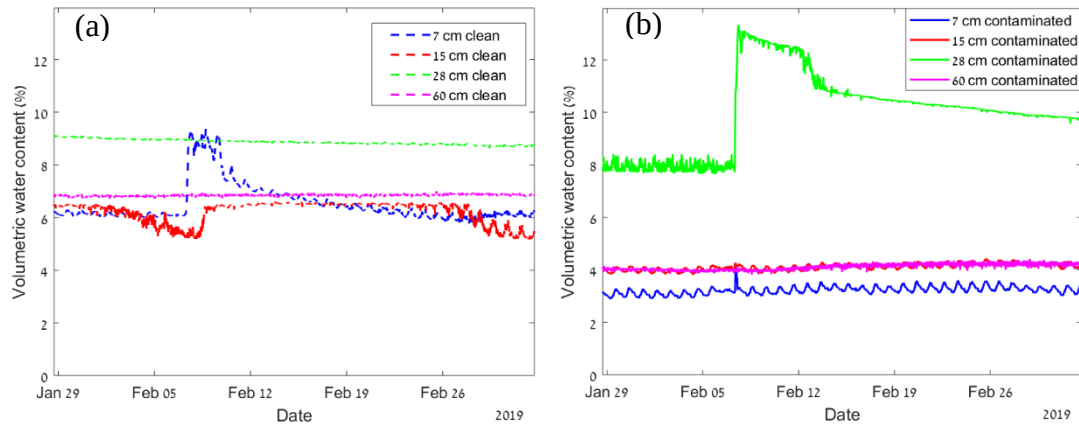


Figure 21: Depth-averaged water content in the downstream station showing the February 7 2019 rain event. (a) Clean section (b) contaminated section.

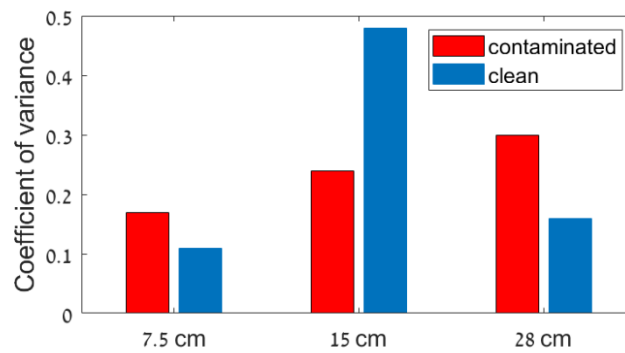


Figure 22: Coefficient of variance of the water content during Feb 2019 rain event.

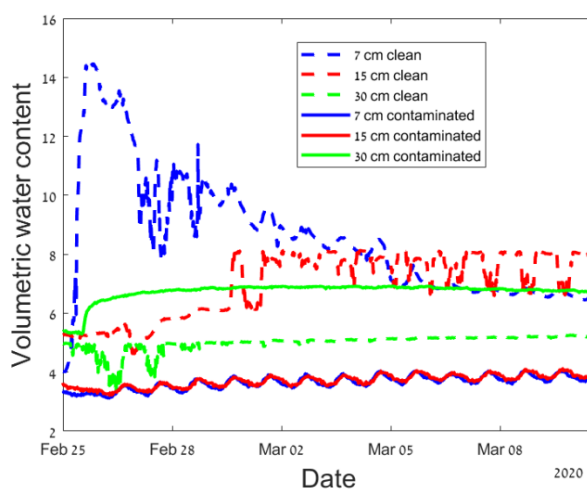


Figure 23: Depth-averaged water content in the downstream station showing the February 25 2020 rain event. Dashed lines show the water content of the clean section while solid lines corresponds to the water content in the contaminated section.

In the clean section, the figure shows an abrupt increase in the water content at depth of 7 cm and a delayed and smaller increase in the 15 cm sensors, as expected. In the contaminated stream, however, the water content in the 7 cm and 15 cm sensors is stable, while only the sensors at 30 cm depth show a fast increase in the water content. This again suggests the existence of fast-conduits of preferential flow that rapidly transport water and solutes to 30 cm depth, where the less permeable gypsum layer impedes flow and cause lateral spread of the water and an increase in the water content.

Figure 24 shows the depth-averaged water contents in the clean (Fig. 24a) and contaminated (Fig. 24b) sections of the downstream station measured during and after the March 15 2020 rain event. As in previous rain events, it is shown that the water contents in the clean stream are much higher compared to the contaminated stream. The irregular pattern in the contaminated stream is evident in this event as well. The highest water content is obtained at 30 cm depth whereas the 15 cm sensors do not show any response, suggesting that water flow bypass them. Estimation of the water depth which penetrated during this event revealed 55 mm in the clean stream versus only 13.7 mm in the contaminated stream. This again shows that in the clean stream about 4 times water penetrated into the soil compared to the contaminated stream. This has substantial implications regarding the water balance in the basin, the amount of available water to plants and the transport of contaminants both in the subsurface and above it.

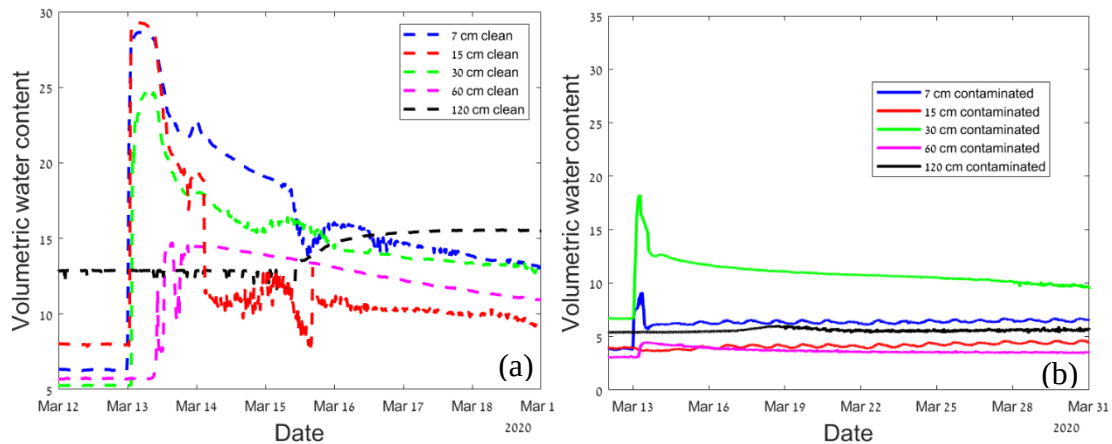


Figure 24: Depth-averaged water content in the downstream station showing the March 13 2020 rain event. (a) Clean section (b) contaminated section.

Figure 25 shows the depth-averaged water contents in the clean (Fig. 24a) and contaminated (Fig. 24b) sections of the upstream station measured during and after the March 15 2020 rain event. Compared to the downstream streams, the infiltration pattern in these streams is much more abrupt, reflecting the coarse texture of the soil in this region. The water in the contaminated stream seems to infiltrate easily into the soil, compared to the downstream station. Indeed, 56 mm of water infiltrated into the soil in the contaminated stream while only 37 mm infiltrated into the contaminated stream.

This has two possible explanations. Observation indicated that the flow in the contaminated stream was greater than in the clean stream due to poor feeding of the clean stream from the upstream. The second reason for the high infiltration in the contaminated stream is that as opposed to the downstream contaminated stream which was heavily oil-contaminated, in the upstream station, only a narrow strip along the cross-section of the stream was contaminated and its hydrophobicity was not as severe as the one in the downstream station.

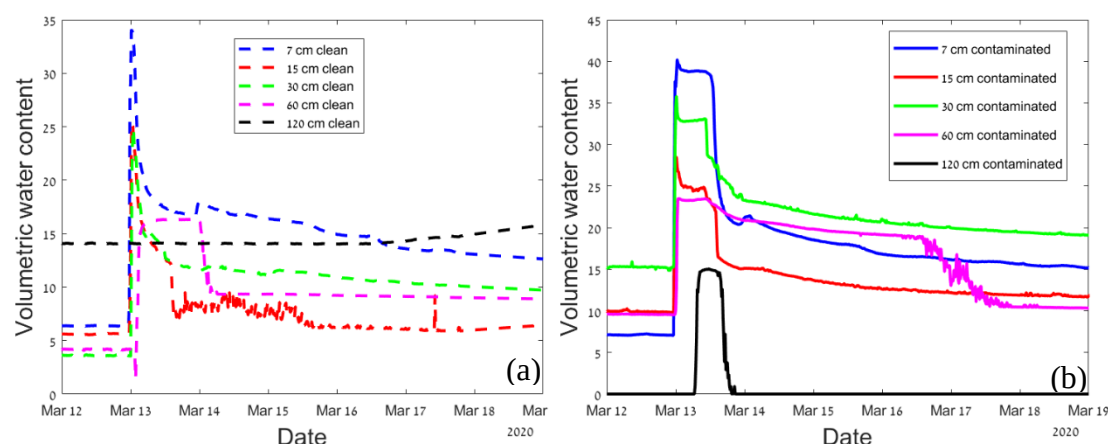


Figure 25: Depth-averaged water content in the upstream station showing the March 13 2020 rain event. (a) Clean section (b) contaminated section.

Figures 26 and 27 show the daily-averaged temperatures for each depth (averaged over all sensors at each depth) through the monitoring period, in the downstream and upstream stations, respectively. The classic seasonal course of temperature is clearly visible. In the downstream section, the temperatures of the sensors of the polluted section are consistently higher than in the corresponding sensors of the clean sensors (except for during the period between July 10- June 16, where an artificial flooding experiment conducted in the contaminated stream by colleagues from Ben Gurion University caused cooling of the contaminated section). The gap in the polluted data between August and October 2019 is due to accidental damage to the sensors.

This results in a difference of between 0.6 to 1 °C in the mean temperature, depending on the depth. In the upstream station, the differences are less pronounced, but still the temperature in the contaminated stream is higher than that of the clean stream most of the time. This results in a difference of 0.4 °C in the mean temperature in the top 30 cm, and 0.2 °C at a depth of 60 cm. This temperature difference may explained by the darker color of the oil-contaminated soil or by the decrease in evaporation from this soil. The smaller spread of oil in the upstream station may explain the small difference in the mean temperature, compared to the heavily-polluted downstream station. Temperature changes induced by oil contamination may have significant effects on animal behavior. This is currently being investigated by the monitoring program operated by the Nature and Park authority in Evrona nature reserve.

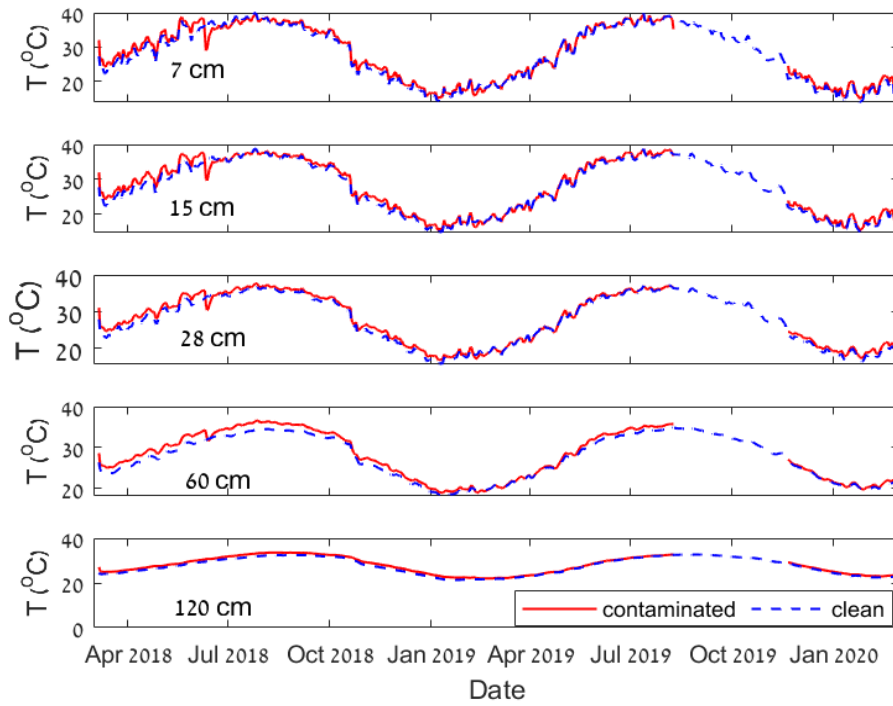


Figure 26: Daily-averaged temperatures measured in the downstream station in the clean and contaminated sections. (a) 7 cm (b) 15 cm (c) 28 cm (d) 60 cm (e) 120 cm. Curves represent averages over all sensors at a specific depth.

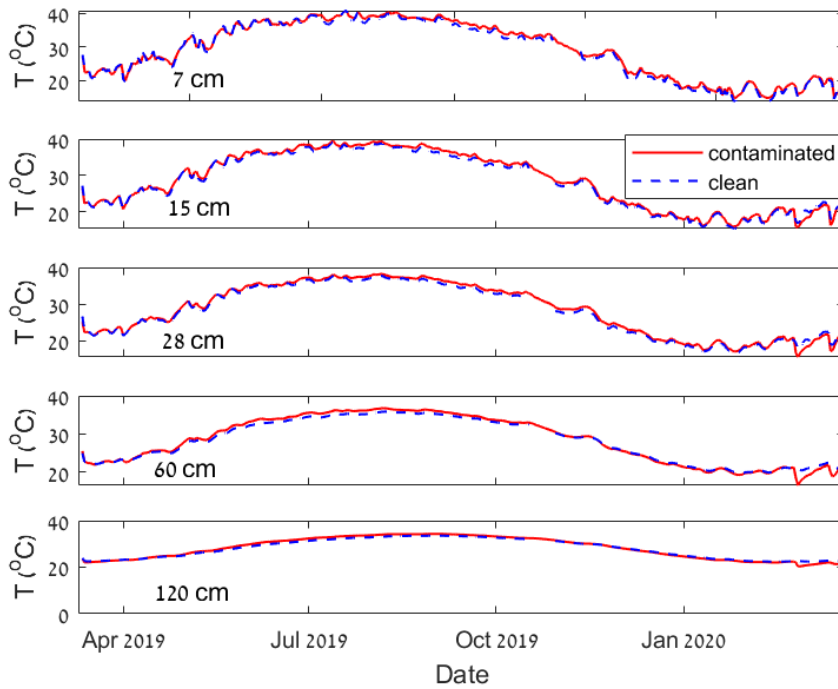


Figure 27: Daily-averaged temperatures measured in the upstream station in the clean and contaminated sections. (a) 7 cm (b) 15 cm (c) 28 cm (d) 60 cm (e) 120 cm. Curves represent averages over all sensors at a specific depth.

The water retention curves of the downstream station and another nearby site (named 2014 control and 2014 clean, for the contaminated and clean samples, respectively) were characterized the Hyprop evaporation method. Figure 28 presents the WRCs of the clean and polluted soil samples obtained by the Hyprop experiment. The fitting parameters by van Genuchten's model (Eqs. 4 and 5) are listed in Table 6. The model predicts the water retention curves well as indicated by the low root mean square errors (RMSEs). The parameter α is related to the inverse of the air-entry value and the parameter n is directly related to the slope of the water retention curve (van Genuchten, 1980). A smaller α indicates a higher air-entry and a high n indicates a uniform soil pore size (Hyun et al., 2008).

The clean soils held more water than the corresponding polluted hydrophobic soils. Roy and McGill (1998) proposed that water retention of wettable soil was higher than in nonwetable soils throughout the suction.

The fitting parameter α , was observed to be higher in the 2014 control and field polluted soils than in the 2014 clean and field clean soils, respectively (Table 6). This finding is in agreement with the results of other studies showing that oil pollution increased α (Hyun et al., 2008; Takawira et al. 2014).

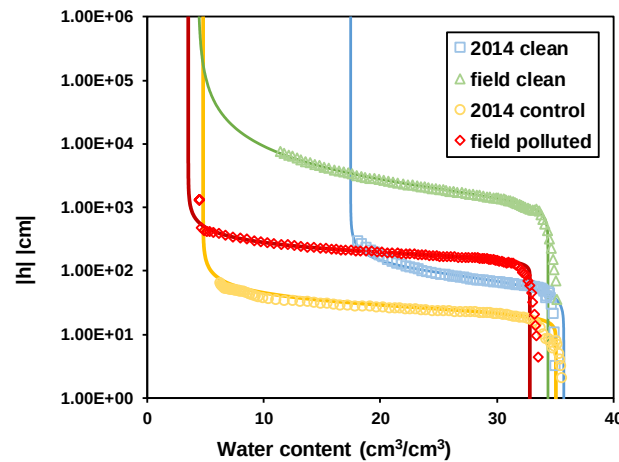


Figure 28: Water retention curves measured by Hyprop evaporation for clean and polluted soil samples incubated in the lab and from the field.

Markers represent measurements, solid curves indicate fitting to van Genuchten's model.

Table 6: Fitting parameters for the van Genuchten's model obtained for the HYPROP data

	2014 clean	2014 control	field clean	field polluted
RMSE_TH	0.0021	0.006	0.002	0.004
RMSE_K	0.1267	0.37	0.17	0.36
α (1/cm)	0.02	0.04	0.01	0.06
n	15.000*	5.73	3.61	10.34
θ_r (cm ³ /cm ³)	0.16	0.07	0.10	0.04
θ_s (cm ³ /cm ³)	0.35	0.35	0.35	0.33
m	0.10	0.66	0.42	0.26
K_s (cm/d)	0.50	0.73	0.71	10.10
τ	-1.76	-1.35	-1.65	-0.78

The root mean squares error (RMSE) is an indication of the mean distance between a data point and the fitted function. RMSE_TH is the root mean square error of retention data. RMSE_K is the root mean square errors of conductivity.

The hydraulic conductivity functions of the clean and polluted soil samples were characterized by the Hyprop evaporation experiments. As presented in Fig. 29, and as can be expected, there is no big difference in hydraulic conductivity between the two clean samples. The values of conductivity in polluted soils are higher than in corresponding clean soils. This result is in agreement with other studies. Takawira et al. (2014) reported that the conductivity increased linearly with the level of hydrocarbon contamination.

In other studies conducted in Evrona, Stavi and Rosenzweig (2019) reported that the nearly saturated hydraulic conductivity was lower in contaminated sediments than in corresponding clean sediments, which is expected as the oil spill decreased the infiltration capacity of the sediments. Gordon et al. (2018) found that the nearly-saturated hydraulic conductivity in the 2014 contaminated site was lower than in clean sediments at the ground surface and at 5 cm depth while it was surprisingly higher than that of non-contaminated soil at 10 cm depth. They explained that this increase could result from an increase in soil aggregation induced by oil pollution. Aggregation changes the soil pore size distribution, increases the fraction of large pores and therefore increases hydraulic conductivity. As shown in Table 6, the saturated hydraulic conductivity (K_s) of the contaminated soil was higher in both polluted soils with respect to the corresponding clean soils. Therefore, it is reasonable to hypothesize that the hydrophobic organic substrates increased the soil aggregation and the fraction of macropores in both samples of 2014 polluted soils.

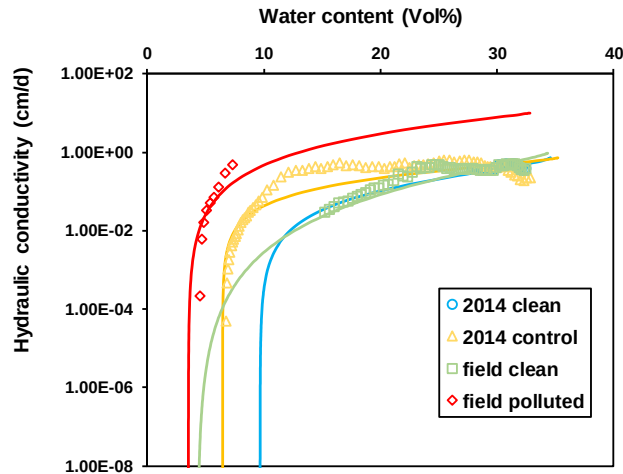


Figure 29: Hydraulic conductivity functions measured by Hyprop evaporation for clean and polluted soil samples incubated in the lab and from the field. Markers represent measurements, solid curves indicate fitting to van Genuchten's model.

Incubation experiments

Soil hydrophobicity

In this study hydrophobicity was quantified using three methods: drop contact angle (DCA), water drop penetration time (WDPT) and molarity of ethanol droplet (MED). Figure 30 presents the results of the WDPT measurements for the 10 treatments. For extremely non-wettable soils (WDPT>3600 s) an arbitrary value of 4000 s was chosen.

It is shown that the control treatments and the 50% saturation treatment did not show any change in their WDPT during the 1.5-year incubation and are classified as extremely non-wettable. All treatments to which nutrients or surfactants were added (2014 20% +Nut+Surf; 2014 20% +Nut, 2014 20%+ Surf and 1975 20% +Nut) and the 20% saturation treatments showed a substantial decrease in their WDPT and are now classified as strongly non-wettable (2014 20% +Sur and 1975 20% +Nut) and severely non-wettable (1975 20%, 2014 20%, 2014 20%+Nut, 2014 20%+Nut+Surf). It is shown that for some of the treatments, hydrophobicity decreases and then increases again. The reason for this is not yet clear and could be related to polymerization of the biodegradation products or secretion of hydrophobic substances by bacteria. While the differences between the WDPT of the four treatments of the 2014 site have narrowed down after one year, the addition of nutrient and surfactant seems to affect the rate of hydrophobicity decrease. The fastest decrease occurred in the 1975 soil with nutrients (1975 20% +Nut) in which the WDPT decreased to 980s after only one month, the 2014 soil with nutrients and surfactant exhibited the second fastest rate and the 2014 soil with nutrients presented slightly slower rate. When only surfactant was added to the wet soil (2014 20%+surf), the WDPT started to decrease only after 4 months, while the addition

of water alone to 20% saturation required a long lag time of 7 and 9 months to induce hydrophobicity reduction in the 2014 and 1975 soils, respectively. It is interesting to note that the 20% saturation treatment performs better than the 50% saturation treatment. This suggests that high water saturation is not needed to efficiently decrease hydrophobicity.

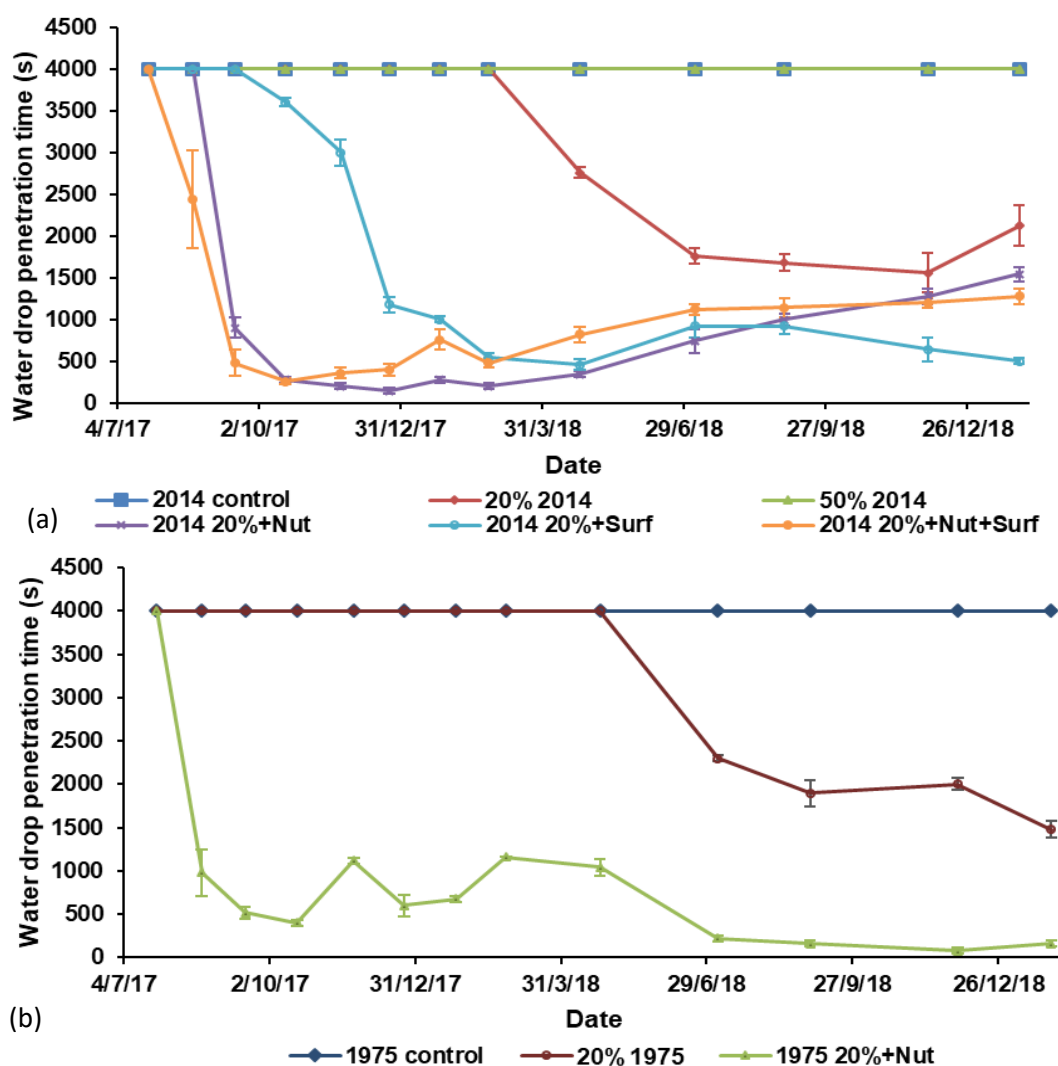


Figure 30: Water drop penetration times (WDPT) for the treatments of the soil from the (a) 2014 spill site (b) 1975 spill site.

Figure 31 presents the results of the MED measurements for all treatments. The results of the MED test show similar trends to the WDPT test: over the 1.5-year incubation a significant decrease in MED occurred in all treatments but the controls and the 50% saturation. At the end of the experiments, the MED values of these treatments were similar to each other but the rate of decrease differed between treatments. The fastest decrease occurred with the addition of nutrients and surfactant, then with nutrients, then with surfactant alone and finally with water (20% saturation). However, the MED test is more sensitive and shows that the hydrophobicity of treatments to which water was added to 50% saturation also decreased. It is interesting to note that the same increase

in hydrophobicity that was observed in the WDPT measurement was also shown in the MED test.

Figure 32 displays the results of the contact angle measurements. Generally, the contact angle of the different treatments decreases during the incubation experiment, but this trend is not as pronounced as in the WDPT and the MED tests and the test exhibits high intra-sample variation as reflected by the high standard deviations.

It is important to note that even though a decrease in the severity of hydrophobicity was demonstrated in all tests; the samples, even after 1.5 year of incubation, still exhibit high degree of hydrophobicity. As hydrophobicity is associated with negative effects, the way to further decrease hydrophobicity should remain a topic of active research.

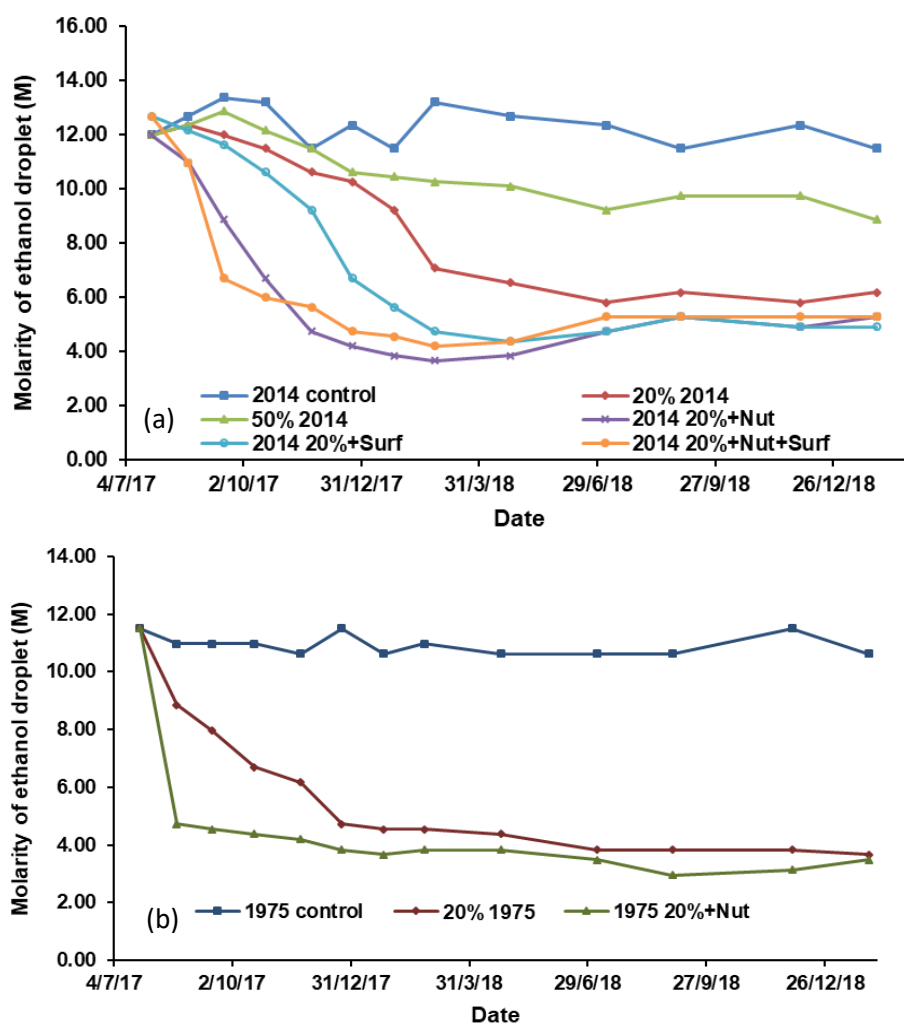


Figure 31: Molarity of ethanol droplet (MED) for the treatments of the soil from the (a) 2014 spill site (b) 1975 spill site.

Figure 33 shows the relation between MED and WDPT for the 2014 and 1975 soils. The results of the MED are positively related to the WDPT after the WDPT started to decrease. The fitted linear relations exhibit a good fit for two of the six treatments (2014 20%, $R^2 = 0.79$; 2014 20%+ Surf, $R^2 = 0.97$). The results for the 2014 treatments are in line with the findings of Takawira et al. (2014) which found a positive correlation in

two oil-contaminated sites in Zimbabwe, albeit of a power function, between the average WDPT and MED. Interestingly, the slopes of the relation for the 2014 and 1975 treatments exhibit the same range of values. MED shows sharper reduction with WDPT for the treatment with the addition of surfactant or nutrients compared to the addition of water alone. When comparing the influence of the addition of water in the 2014 and 1975 soil, it is found that for the same WDPT value, the MED value of the 2014 soil is greater than that of the 1975 soil. These show that although the WDPT and MED both quantify hydrophobicity and are positively correlated, in the case of oil-induced soil hydrophobicity, a unique, universal relation between the two measures is not available. This is in agreement with Doerr (1998) who found high correlation between the two measures, but no consistent relation.

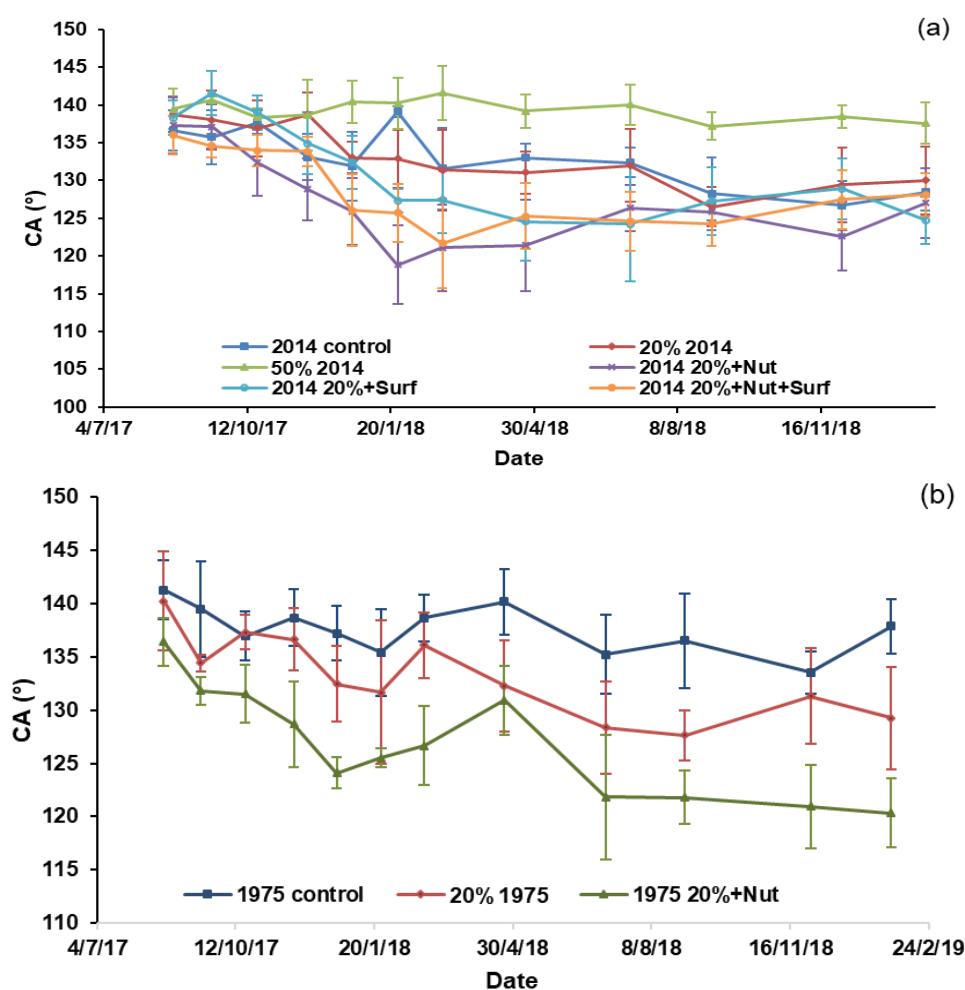


Figure 32: Drop contact angle for the treatments of the soil from the (a) 2014 spill site (b) 1975 spill site.

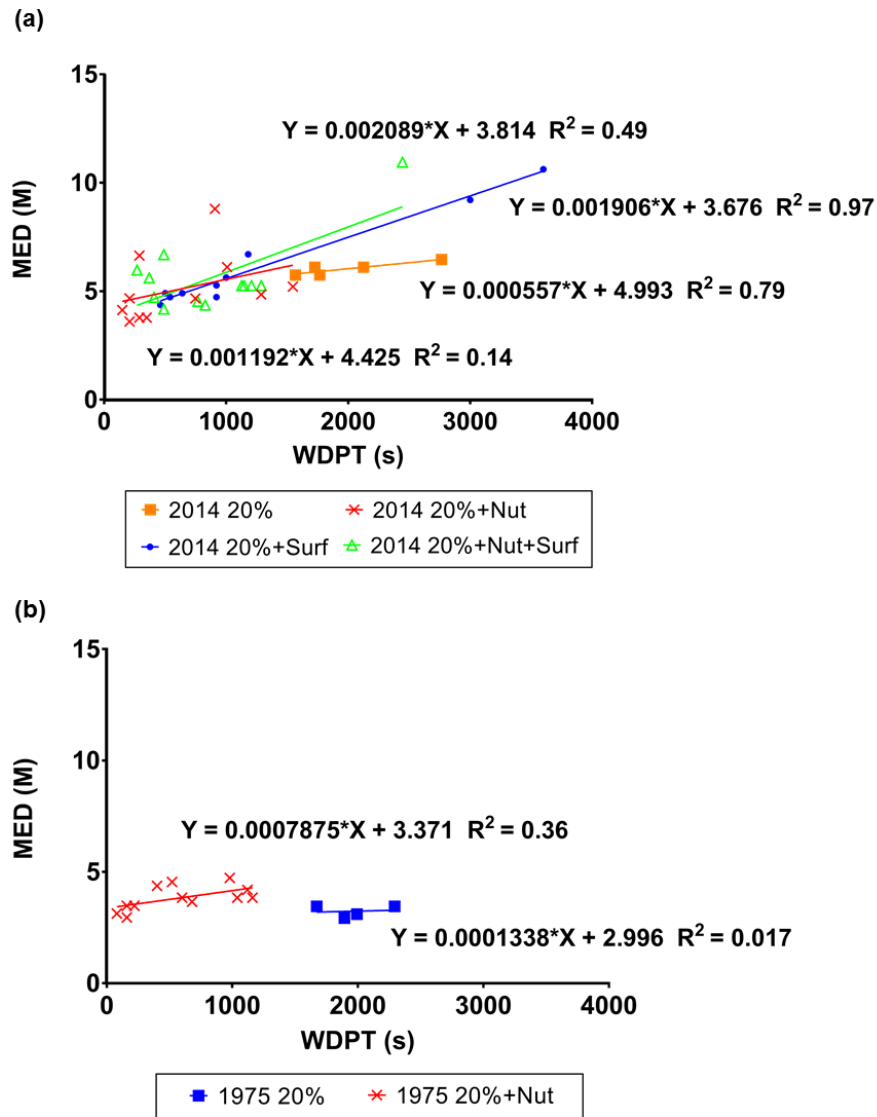


Figure 33: Relation between WDPT and MED for (a) 2014 spill site (b) 1975 spill site.

Measurements of unsaturated hydraulic properties

Water retention of the 7 treatments of the 2014 soil were measured using the dew point method and fitted to the van Genuchten's relation (van Genuchten, 1980; Eq. 4). The measured curves are shown in Figure 34.

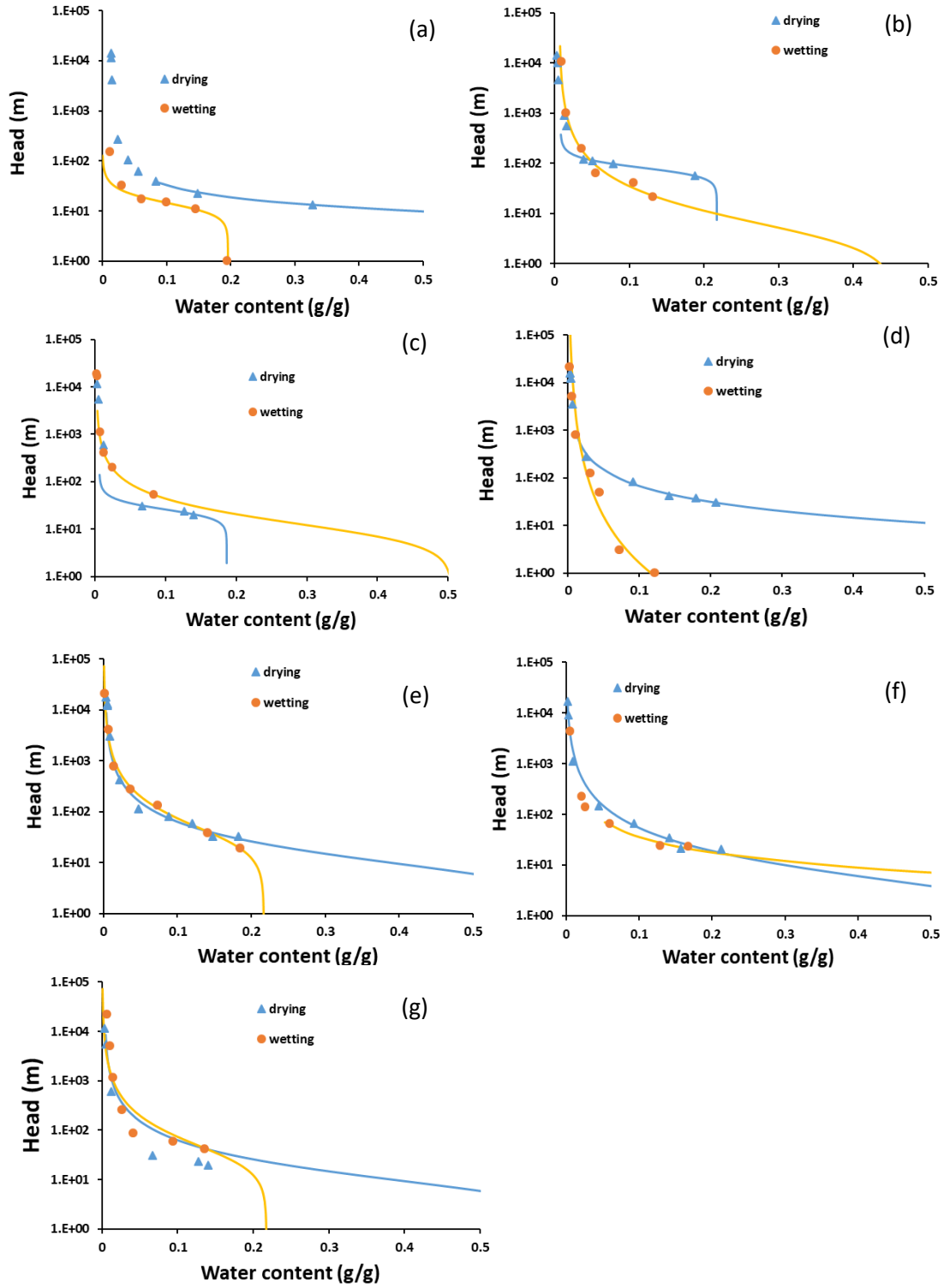


Figure 34: Water retention curves for the 2014 spill site (a) clean soil (b) control (c) 50% saturation (d) 20% saturation (e) 20% + Surf (f) 20% +Nut (g) 20% +Nut +Surf. Markers represent measurements, slid curves indicate fitting to van Genuchten's relation.

As the potential obtained from the dew-point test is the sum of the matric potential and the osmotic potential, the contribution of the osmotic potential was approximated by measuring the electrical conductivity of a saturated paste and evaluating the osmotic potential by the following relation,

$$\Phi_o = 0.036 EC \quad (1)$$

where Φ_o is the osmotic potential in MPa and EC is the electrical conductivity in ds/m. The calculated osmotic potential ranged between 0.06 MPa in the 1975 samples to 0.13 MPa in the 2014 samples to which a nutrient solution was added. As dew-point measurements are considered accurate at potentials higher than 0.5 MPa (where the measurement error is lower than 10%), the contribution of the osmotic potential at this range is rather small.

Figure 18 shows that the retention of most samples is quite similar. However, the samples reveal intricate hysteretic behavior. While a classic hysteresis is observed in the clean soil, control and 20% saturation where the drying curve is wetter than the wetting curve, in the 50% saturation sample, the opposite behavior is observed. This calls for additional tests, which will be performed next year. The treatments that contain nutrients and/or surfactants, show very small hysteresis. This may be due to the addition of surfactants and the secretion of biosurfactants, which are known to reduce hysteresis. The fitted parameters of the van Genuchten's relation are listed in Table 7. It shows that the residual saturation is very low, as can be expected for sandy soils. It is also shows that n lies in the range of 1.3-4.8, which generally also characterizes coarse-grained soils and high variation is observed in the value of α . As the dew-point method characterizes the retention at high potentials while α characterizes the air entry pressure, this parameter cannot be fitted properly with this measurements.

Table 7: Fitted parameters to van Genuchten's relation

Sample	α (1/m)	n	θ_r	α (1/m)	n	θ_r
	drying			Wetting		
2014 clean	1.45	2.48	0.017	0.076	3.62	N.A
2014 control	0.013	4.8	0.008	0.28	1.69	0.006
2014 20%	0.72	1.92	0.002	140	1.3	NA
2014 50%	0.04	4.44	0.007	0.11	2.01	0.002
2014 20% Surf	0.168	1.82	0.002	0.033	1.77	NA
2014 20% Nut	0.38	1.67	NA	5.09	2.01	0.004
2014 20% Nut + Surf	0.098	1.89	0.001	0.017	4.76	0.014

Evaluation of alkane biodegradation

Chromatograms of GC-MS analyses obtained for the hydrocarbons extracted from the contaminated soils are shown in Figure 35. The chromatogram of the untreated contaminated 2014 soil from July 2017 was observed to contain n-alkanes ranging from n-C₁₄ to n-C₃₄, with n-C₂₀ as the main peak (Fig. 35a). After 1-year incubation, the control shows a slightly modified chromatogram where C₁₄-C₁₅ disappeared and the main peak shifted to n-C₂₂ as (Fig. 35b). Figure 35c indicates the changes after 1-year

incubation in the presence of nutrients (2014 20% + Nut), showing the almost-complete removal of lower carbon-numbered alkanes and shifting the main peak to n-C₃₂. The chromatogram of the untreated contaminated 1975 soil from July 2017 is typical of weathered oil containing alkanes ranging from n-C₁₅ to n-C₃₄, with the main peak at n-C₂₁ (Fig. 35d). As in the 2014 soil, after 1-year incubation, the chromatogram of the 1975 control (Fig. 35e) shifted slightly towards higher-weight n-alkanes showing weathered oil without the C₁₃-C₁₈ n-alkanes and only small Pristane (Pr) and Phytane (Ph) peaks. Incubation of this soil with nutrients substantially accelerated the biodegradation of the oil, showing after 1-year incubation, the complete removal of the low-weight alkanes and domination by the high-weight alkanes with the main peak at n-C₃₁ (Fig. 35f).

Microbial degradation rate is commonly characterized by ratios such as n-C₁₇/Pr and n-C₁₈/Ph (Wang and Fingas, 2003) and the ratio between low-weight Σ (n-C₁₄-n-C₂₁) and high-weight molecular alkanes Σ (n-C₂₂-n-C₃₄) (L/H). In the early stages of biodegradation, the removal of more biodegradable alkanes occurs, with the conservation of more degradation-resistant isoprenoids (pristane and phytane), resulting in an increase in Pr/n-C₁₇ and Ph/n-C₁₈ ratios (Barakat et al., 2002). However, in the late stages of biodegradation, Ph and Pr degrade as well, leading to a decrease in Pr/n-C₁₇ and Ph/n-C₁₈ ratios (Frontera-Suau et al., 2002). The evolution of these ratios along the first year of the incubation experiment for the different treatments is presented in Figure 36. As in most of the treatments, Pr and Ph were completely degraded during the first year of incubation, results are not shown for the rest of the incubation experiment. Obviously, during the incubation experiment, Pr/n-C₁₇ and Ph/n-C₁₈ remained almost constant in the 2014 and 1975 controls, whereas these ratios changed significantly in the treated samples. Although during the first six months of the incubation experiment, degradation was more pronounced in the samples with the addition of nutrients or surfactants, during the next months of the experiment, significant degradation of the hydrocarbons was observed also in the samples supplemented with water to 20% saturation. The change of alkanes in the different treatments is concomitant with the results of the hydrophobicity shown earlier. The decrease in the L/H ratio of the control treatments reflects the degradation of the low-weight alkanes observed in the chromatograms (Fig. 35) and can be attributed, at least in part, to the intensive mixing and aeration throughout the incubation experiment.

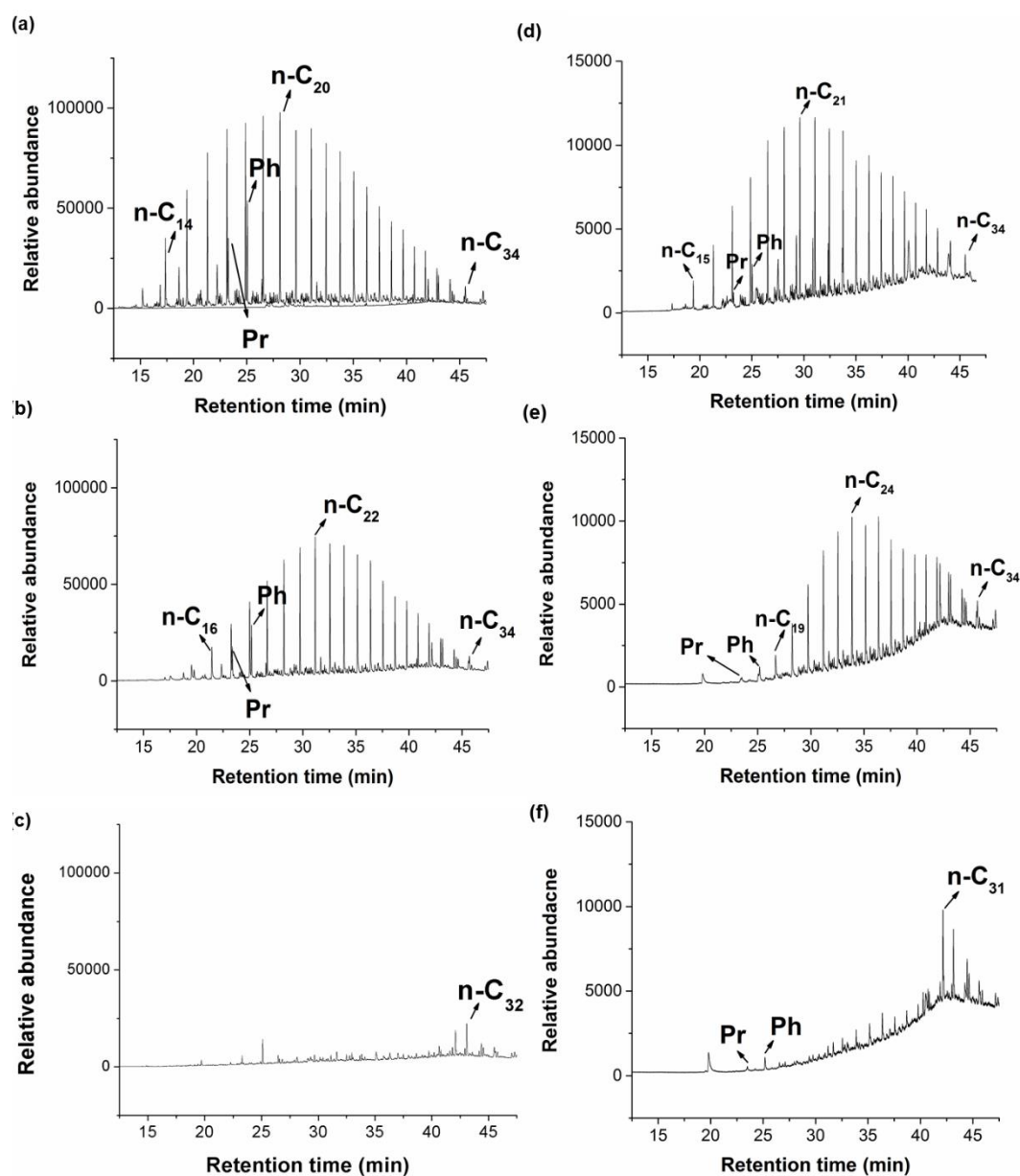


Figure 35: GC-MS chromatograms of oil extracted from the incubated soils. (a) Control 2014 soil (July 2017) (b) Control 2014 soil (July 2018) (c) Treated soil 2014 20% + Nut (July 2018) (d) Control 1975 soil (July 2017) (e) Control 1975 soil (July 2018) (f) Treated soil 1975 20%+ Nut (July 2018)

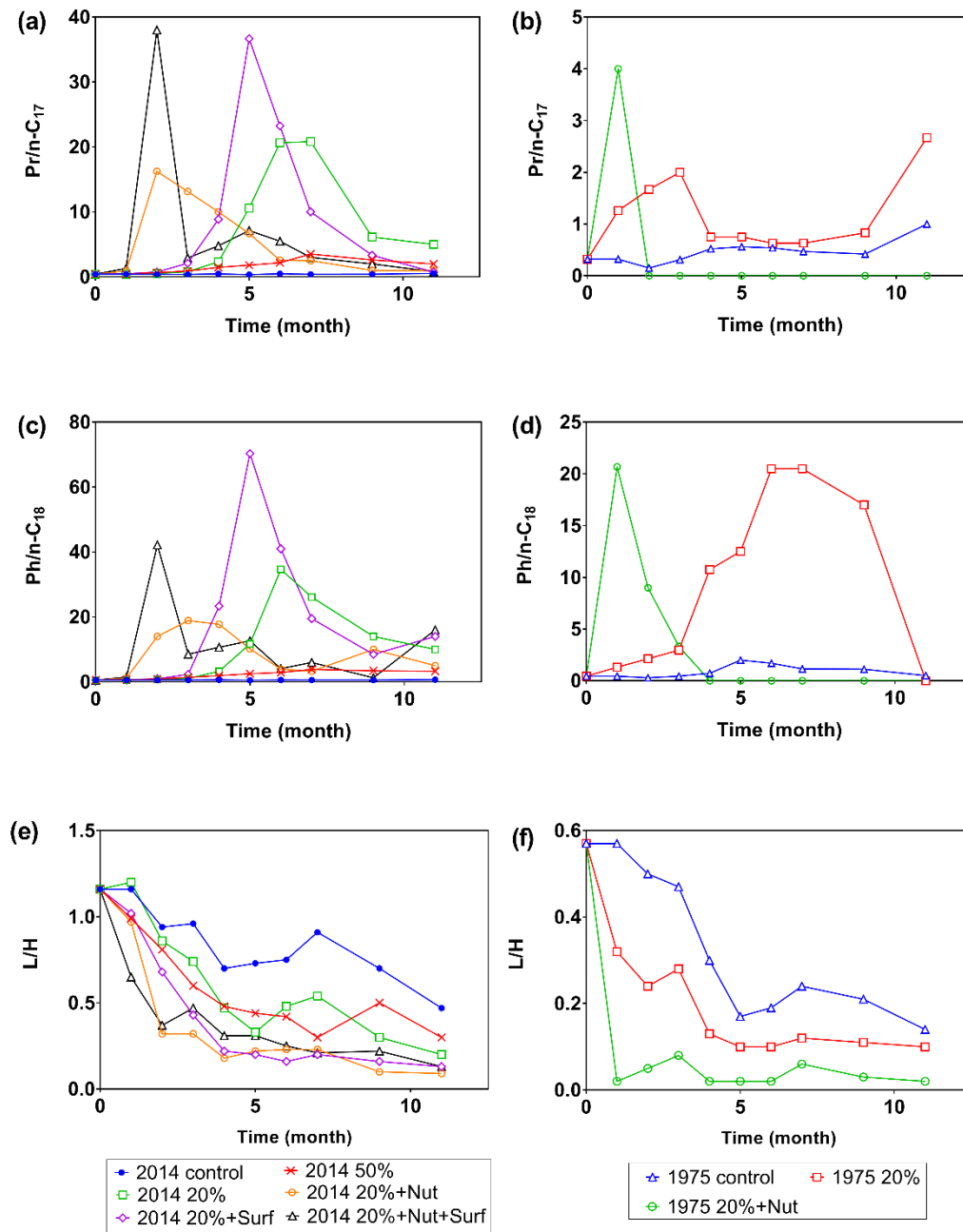


Figure 36: Changes in Pr/n-C₁₇, Ph/n-C₁₈ and L/H ratios along the incubation experiments in soil samples treated under different conditions. (a) Pr/n-C₁₇, 2014 (b) Pr/n-C₁₇, 1975 (c) Ph/n-C₁₈, 2014 (d) Ph/n-C₁₈, 1975 (e) L/H, 2014 (f) L/H, 1975

Figure 37 presents the TPH (DRO+ORO; EPA-8015) values for all the treatments during the entire incubation period. For the 2014 soils, the fastest decrease of TPH occurred in the treatment with the addition of nutrients and surfactant, then with the addition of nutrients and then with the addition of surfactant alone. The 20% saturation treatment exhibits the slowest rate of these treatments, but this is somewhat misleading as sampling was not conducted often enough to get a reliable measure of the rate. The 50% saturation treatment showed a lag time of one year, but then its TPH reduced significantly, at a rate comparable to the rate of the other treatments.

For the 1975 contaminated soil, during the 1.5-year incubation period, the TPH decreased by 82.6% with the addition of nutrients (1975 20%+Nut) and by 41.7% when only water was added (1975 20%). We note that in both of the 2014 and 1975 controls, a small decreases in TPH (17.6% and 7.7%, respectively) were observed.

Similarly to the results of WDPT and MED tests, at the end of the incubation, the differences between the 2014 treatments in terms of TPH removal were rather small (difference of 18% between the extream values and $P = 0.48$). Further, the level of TPH removal between treatments (2014 20% + Surf > 2014 20% + Nut + Surf > 2014 20% + Nut > 2014 20%) correlated with the reduction of MED and WDPT at the end of the experiment.

The susceptibility to microbial degradation generally decreases in the following order: n-alkanes > branched alkanes > low-weight aromatics > cyclic alkanes (Leahy and Colwell, 1990). The biodegradation rate is slowed once the removal of easily-degraded alkanes and lower-molecular-weight aromatics complete. Some of the high-molecular-weight polycyclic aromatic hydrocarbons (PAHs) may not be degraded at all (Atlas and Bragg, 2009). Wrenn et al. (2006) found that nutrient demand for oil-spill bioremediation decreased as the oil biodegradation progresses. They suggested that after the easily degradable n-alkanes are almost completely depleted, nutrients are no longer the limiting factor for the biodegradation rate of the less degradable organic compounds. Although in the present study aromatic fractions were not analyzed, it is reasonable to hypothesize that in our experiment, when the more resistant hydrocarbons started to degrade, the addition of nutrients did not accelerate the biodegradation rate. Biosurfactant, on the other hand, could continuously increase the bioavailability of the hydrocarbons to microorganisms. This may explain the observation that the TPH of 2014 20% + Nut + Surf and 2014 20% + Nut treatments decreased very fast at the first months but did not exhibit the highest TPH removal at the end of the incubation. This may also contribute to the lowest hydrophobicity in 2014 20%+Surf treatment at the end of the incubation, as indicated by both WDPT and MED tests.

In the high saturation treatment (2014 50%), the long lag time can be attributed to the development of anaerobic conditions (developed in spite of the stirring) which could slow down the growth and activity of the microorganisms compared to the other treatments in which water content was lower. We hypothesize that over this prolonged period of incubation, anaerobic microorganisms adapted to the high-saturation conditions and started to degrade the hydrocarbons. This corresponds with the results of hydrophobicity, where the slowest and mildest hydrophobicity reduction was obtained for the 2014 50% treatment.

Altogether, even with ~40% reduction in TPH for the 2014 treatments and with a 41.7% and an 82.6% reduction for the 1975 20% treatment and 1975 20% + Nut treatments, soils remained severely water repellent, demonstrating the permanence of hydrophobicity.

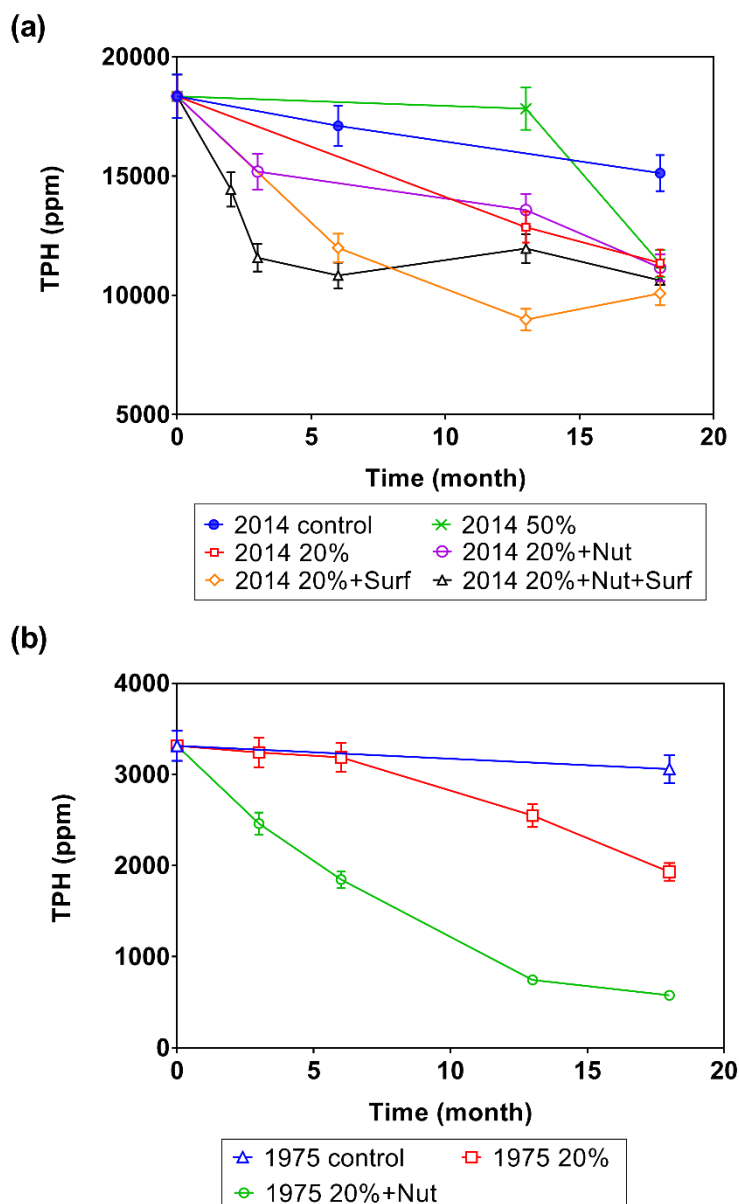


Figure 37: TPH values throughout the incubation experiment from the (a) 2014 spill site (b) 1975 spill site. Error bars present the analytical standard error associated with the measurements.

Quantification of microbial oil degraders

The real-time PCR quantification of the *alkB*, *nahAc*, and *dmpN* genes is shown in Figure 38. The number of copies of the *alkB* gene was below the lowest limit of the standard curve ($< 1.6 \times 10^3$ copies/ g dw) in the control treatment of the 1975 soil at the beginning and significantly increased after 1.5-year incubation in all the 1975

treatments (1975 control, 1975 20%, and 1975 20% + Nut), ranging from 3.65×10^5 to 3.57×10^6 copies per gram dry soil. The 1975 20% + Nut treatment presents the highest number of copies at the end of the incubation, followed by the control soil and the 1975 20% soil. The number of copies of *nahAc* and *dmpN* genes was below the detection limit (1.6×10^4 copies/g) in the 1975 soil samples.

A dramatic increase in the number of copies of the *alkB* gene was observed in all the 2014 treatments after 1.5-year incubation, ranging from 4.34×10^5 to 1.06×10^7 copies per gram dry soil, 10 to 200-fold greater than the 7.85×10^4 copies per gram dry soil obtained in the initial stage of incubation. At the end of the incubation, the order of the number of copies of the different treatments is as follows: 2014 50% > 2014 20% > 2014 20% + Nut + Surf > 2014 20% + Surf > 2014 20% + Nut. The Number of copies of the *alkB* gene in the 2014 50% treatment was found to be significantly greater than in the 2014 20%, 2014 20% + Nut, 2014 20% + Surf and 2014 20% + Nut + Surf treatments by a one-way ANOVA analysis ($P < 0.05$). The treatments of the 2014 control and the 2014 clean soil presented higher abundance of the *alkB* gene than the soil treated with nutrients and surfactants at the end of incubation.

At the end of incubation, the maximum number of copies of *nahAc* gene was observed in the 2014 50% water saturation soil, 100 to 1000-fold greater than in the other treatments. This followed by the 2014 20% + Nut soil, and then the 2014 20% and 2014 20% + Nut + Surf, with the 2014 20% + Surf treatment exhibiting the lowest number of copies. The copies of *dmpN* gene were below the detection limit ($< 1.6 \times 10^3$ copies/g) in the 2014 control and in the clean soil samples. After the incubation, the abundance of *dmpN* gene increased substantially in all the soils treated with water, nutrients, and biosurfactants while the number of copies of the clean and control soils remained below the detection level. The 2014 50% treatment presented the highest abundance of *dmpN* gene, 10 to 50-fold greater than the values of the other treatments, where the 2014 20% + Nut had the second-highest number of *dmpN* copies. Statistical analysis (ANOVA) showed that the numbers of copies of the *nahAc* and *dmpN* genes in the 2014 50% treatment were significantly greater than in the 2014 20%, 2014 20% + Nut, 2014 20% + Surf treatments and in the 2014 20% + Nut + Surf treatments ($P < 0.05$).

As the alkane monooxygenase encoded by the *alkB* gene involves an initial activation step of aerobic aliphatic hydrocarbon metabolism (Jurelevicius et al., 2013), it primarily targets short and medium alkanes (C5~C16) (Van Beilen and Funhoff, 2007). The naphthalene dioxygenase is not only involved in the degradation of naphthalene but also mediates degradation of phenanthrene, anthracene, dibenzothiophene, fluoranthene, and methylated naphthalene thus the *nahAc* gene can be used as a marker for PAH degradation (Park and Crowley, 2006). The phenol hydroxylase encoded by the *dmpN* gene can use NADPH as the electron donor to oxidize phenol to catechol (Nurk et al., 1991). The abundance of these genes could indicate the biodegradation potential of light monocyclic aromatics in contaminated sites (Baldwin et al., 2008; Wang et al., 2010).

The overall number of copies of the *alkB* and *nahAc* genes were observed to be lower in the soil treated to 20% water saturation, with nutrients or/and biosurfactants than in

the control soil at the end of the incubation. Firstly, the gene numbers could increase significantly in the first few months and then decreased in the treated 2014 soils, thus presented lower numbers than in the control soil. One possible explanation of the reduction of hydrocarbon degraders is the lack of nutrients for the metabolism of the bacteria. Bacteria could die and release DNA into the soil, which can then be degraded (with the rate depending on the soil's moisture content and organic content; Sirois and Buckley, 2019). Therefore, DNA could be easily degraded in the soils treated to 20% water saturation, with nutrients or/and biosurfactants, resulting in a low concentration of DNA within these soils. Secondly, the fraction of active alkane-degrading bacteria may have been larger in the treatments with the additions of water, nutrients, and biosurfactants than in the control soils. The active role of the hydrocarbon-degrading bacteria needs further exploration by quantifying the gene expression.

The results reveal that the maximum abundance of *alkB*, *nahAc*, and *dmpN* genes was found in the 2014 50% soil at the end of the incubation. This is in line with the hypothesis that hydrocarbon-degrading microorganisms slowly adapt to the high-saturation conditions during the long incubation time and start to grow after a prolonged lag time while degrading TPH and reducing its concentration (Fig. 37). Liu et al. (2015) found that the number of copies of *alkB* shows a positive correlation with TPH and the concentration of alkanes (C33~C40), and the abundance of *Nah* is negatively correlated with TAH and 16 targets PAHs, thus they concluded that soil with fewer alkanes exhibited less *alkB* abundance (Liu et al., 2015). According to the results, at the end of the incubation, the concentration of TPH in the 2014 50% treatment was close to the TPH of the other 2014 treatments. A strong positive correlation ($R^2 = 0.85$) between the numbers of copies of *alkB* gene and TPH was found in the 2014 soil samples at the end of the incubation experiment when the 2014 50% treatment is excluded (Fig. 39). As TPH value reflects the contribution of the entire mixture of hydrocarbons of crude oil, the amounts of individual alkane and aromatic compounds are unknown. It was hypothesized that at the end of the incubation, among the 2014 soil samples, the 2014 50% soil is the most alkane-rich (Fig. 36) and contains the smallest fraction of aromatic compounds, resulting in the highest *alkB* and *nahAc* abundance. Due to the lack of data, whether the enrichment of the hydrocarbon-degradation functional genes occurred at the same time the TPH removal started (after a lag period), or at another time cannot be inferred.

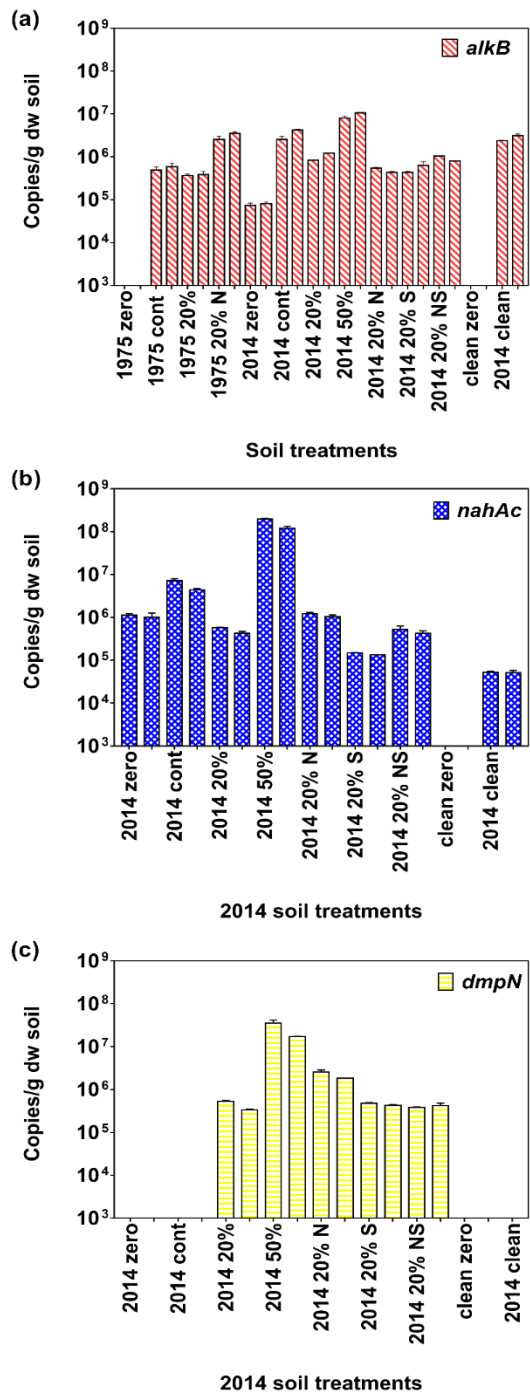


Figure 38: The number of copies of (a) *alkB*, genes in 1975 and 2014 soil samples, (b) *nahAc* and (c) *dmpN* genes at the 2014 samples. The DNA extraction used in qPCR was conducted in duplicates for each soil sample. Values represent means $\pm$ standard deviation for duplicates of each DNA extract. The empty bars mean that the number of copies was below 1.6×10^3 copies/g.

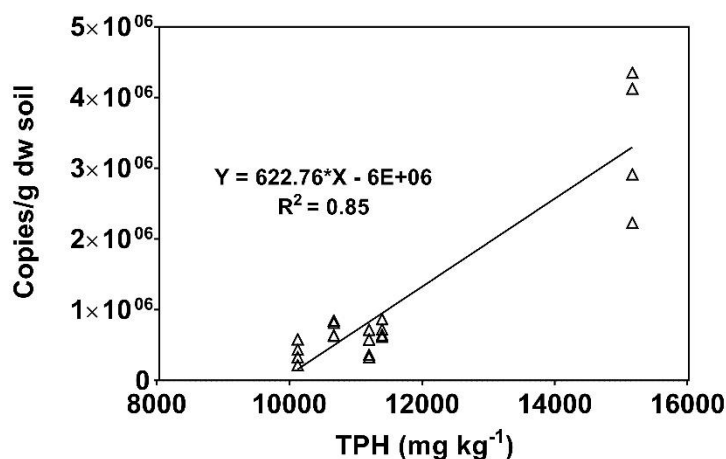


Figure 39: Correlation between the abundance of *alkB* gene and TPH at the end of the incubation

Active degraders obtained by the MPN assay showed that after 6-month incubation alkane and aromatic degraders exist in the soil in all treatments, even in the clean soil (Fig. 40). The number of alkane and aromatic degraders in these treatments did not show a significant increase over the control soil for 2014 soil samples. This is in agreement with the results of the quantification of *alkB* and *nahAc* genes discussed before. Townsend et al. (2000) found that in a crude oil-contaminated coastal wetland, no significant differences were observed in the populations of aliphatic degrading or PAH-degrading microorganisms quantified using the MPN method after two inorganic nutrients treatments. As only ~1% to ~10% of the total microorganisms in the soil can be cultivated, the number of non-cultivable hydrocarbons degrading microorganisms may have been increased without an increase in the MPN which only quantifies bacteria which can grow in the Bushnell Haas (BH) medium. The real-time PCR, on the other hand, quantifies the total number of degraders that remained in the soils, including the dead cells.

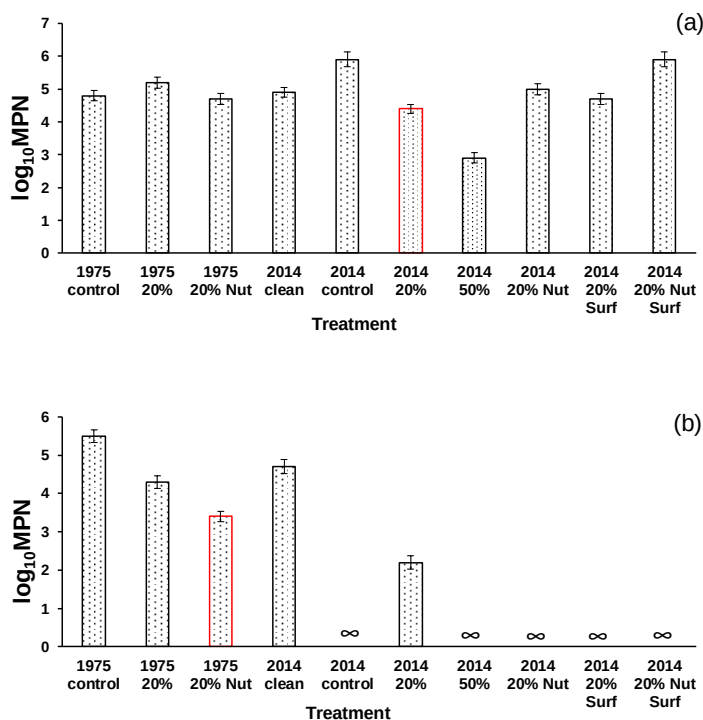


Figure 40: Most-probable-number (MPN) for the (a) Total alkane degraders; (b) Total aromatic degraders in the soils. Samples were taken in March 2018, 6 months after the beginning of the incubation experiment. Red bars indicate statistically unreliable data. The 'infinity' signs denote that the most probable number is higher than the upper limit of the test ($>10^6$).

Assessment of biosurfactant producers

Screening for biosurfactant production activity and characterization of the isolates

The characteristics of oil spreading activity, emulsification activity and surface tension of the promising biosurfactant-producing strains are summarized in Table 8. Ideally, the oil displaced area would be positively correlated with the concentration of biosurfactants in the test solution (Thavasi et al., 2013). In this study, the oil spreading assay was used as a qualitative study to check the production of biosurfactant in the cell-free broth. From this preliminary screening, strains which presented a positive oil spreading activity were donated as NS strains (NS1, NS2, NS3, NS5, NS6, and NS8) and strains which were shown to be negative in the oil spreading assay were donated as CT strains (CT7, CT9, CT10, CT11, CT12, and CT14).

The results of the emulsification activity were in agreement with that of the oil spreading assay. It was hypothesized that the biosurfactant presented in the cell-free broth will emulsify the hydrocarbons (Thavasi et al., 2013). The NS strains which presented a positive activity in the oil spreading assay were also positive in the emulsification assay, with the E24 ranging from 42.4% to 51.9%. Isolated marine biosurfactant-producing bacteria screened by Satpute et al. (2008) displayed high emulsification activity ($> 30\%$) and emulsification of kerosene by Gram-negative bacteria MM73b and *Acinetobacter* spp. MM74 was up to 68 %. In their study, the

isolates which were found to be positive (>30%) in the E24 assay were also found to be positive in other biosurfactant screening assays. This confirms that biosurfactant production can be detected through the E₂₄ assay.

Table 1: Biosurfactant screening assay

<i>Isolate code</i>	<i>Oil Spreading</i>	<i>E₂₄ index</i>	<i>Surface tension (mN/m)^a</i>	<i>Surface tension (mN/m)^b</i>
NS1	+	48.2% ± 0.5%	41.86 ± 0.03	27.70 ± 0.04
		(+)	(++)	(++++)
NS2	+	44.4% ± 0.9%	43.70 ± 0.04	28.92 ± 0.04
		(+)	(++)	(++++)
NS3	+	51.9% ± 0.4%	44.11 ± 0.07	27.56 ± 0.08
		(++)	(++)	(++++)
NS5	+	48.2% ± 0.5%	43.19 ± 0.08	27.67 ± 0.04
		(+)	(++)	(++++)
NS6	+	48.2% ± 0.7%	44.10 ± 0.03	28.82 ± 0.03
		(+)	(++)	(++++)
NS8	+	51.9% ± 0.2%	41.81 ± 0.05	27.65 ± 0.02
		(++)	(++)	(++++)
CT7	-	-	56.40 ± 0.09	/
			(+)	
CT9	-	-	56.76 ± 0.02	/
			(+)	
CT10	-	-	55.51 ± 0.03	/
			(+)	
CT11	-	-	56.15 ± 0.04	/
			(+)	
CT12	-	-	55.39 ± 0.07	/
			(+)	
CT14	-	-	54.22 ± 0.05'	/
			(+)	
PAO1	+	63% ± 0.9%	40.03 ± 0.05	29.61 ± 0.03
		(+++)	(++)	(++++)
Neg	-	-	68.13 ± 0.03	64.06 ± 0.07
			(-)	(-)

a- The experiment was conducted with BH cell-free culture broth with hexadecane as a carbon source

b- The experiment was conducted with SW medium cul cell-free culture broth with glycerol as a carbon source

PAO1.- Biosurfactant producing strain *Pseudomonas aeruginosa* PAO1 (Müller et al., 2010). Neg- The result of distilled water used as a negative control

Oil spreading: '+'- positive oil spreading activity; '-'- no oil spreading activity.

E₂₄ index: '++++'- E₂₄ > 60%; '++'- E₂₄ = 50% - 60%; '+'- E₂₄ = 40% - 50%; '-'- no emulsification activity.

Surface tension: '++++' surface tension < 30 mN/m; '+++' surface tension 30 - 40 mN/m; '++' surface tension 40 - 50 mN/m; '+' surface tension 50 - 60 mN/m; '-' – no surfactant activity.

Biosurfactants can lower the surface tension and increase the solubility of organic compounds, thus increase their bioavailability and enhance hydrocarbon removal (Joy et al., 2017). As shown in Table 8, the surface tension assay of the BH cell-free broth displayed a reduction from ~68 to ~43 mN/m in the surface tension of the NS strains while a reduction from ~68 to 55 mN/m in the surface tension of the CT strains. The SW cell-free culture broth reduced the surface tension from ~68 to 28 mN/m in the NS strains. A highly-active surfactant can lower the surface tension of water from 72 to 35 mN/m (Mulligan, 2005).

Rhamnolipids typically produced by *Pseudomonas* are mixtures containing different structural homologs (Déziel et al., 1999). Perfumo et al. (2006) found that the carbon source affected both the quantity and the structures of biosurfactants. The variety of rhamnolipids can dramatically decrease when produced on hexadecane compared to water-soluble glycerol (Perfumo et al., 2006). This may explain why the SW cell-free broth with glycerol as a carbon source yielded ~59% surface tension reduction with respect to a negative control while the BH broth with hexadecane as a carbon source yielded only ~37% reduction for the same strains.

The components of the biosurfactant produced by strains in this study are not known to us and could be characterized in the future. Due to the lack of OD measurement, we cannot quantify the production of biosurfactant per cell. This should be investigated in future work.

Characterization of the biosurfactant-producing isolates by 16S rRNA sequencing

Based on the sequence analysis, all the isolates were identified as members of *Pseudomonas*. The 16S rRNA sequences of the NS isolates were aligned to the reference sequences found from BLAST. Fig. 28 presents the relative position of isolates in the neighbor-joining phylogenetic tree. Biosurfactant produced by one reference strain *P. aeruginosa* PG1 (Fig. 41), which is closely related to strains NS2 and NS5, presented 100 % emulsification activity in crude oil and 83 % for hexadecane (Saisa-Ard et al., 2014). In their study, the surface tension in the culture broth was continuously reduced from 51.8 mN/m to 29.6 mN/m in 3 weeks. This came along with a simultaneous degradation of ~65 % of the crude oil that was in the medium. This implies that *P. aeruginosa* PG1 can produce biosurfactants while utilizing the crude oil components like a carbon source. Although the surface tension and the degradation of hexadecane during the experiment was not monitored, it is reasonable to conjecture that the isolates with strong emulsification activity and surface tension reduction activity have the potential to be applied for oil degradation.

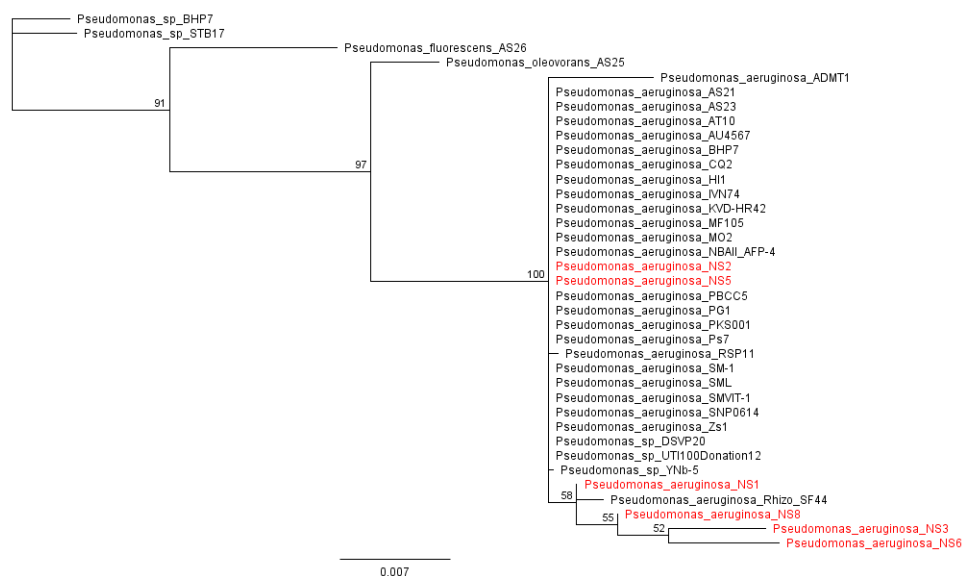


Figure 41: Phylogenetic tree based on the 16S rRNA gene sequencing of the isolated biosurfactant-producing strains obtained using the neighbor-joining method. Scale bar equals approximately 0.005 substitutions per nucleotide position.

Summary and conclusions

The objective of this study is to investigate the persistence and hydrological effects of oil-induced soil hydrophobicity in Evrona by combining field monitoring, laboratory incubation experiments, measurements of soil hydraulic properties and microbiological assays.

Two monitoring stations were installed; one in the upstream of the Evrona basin and the other in its downstream. The stations continuously monitor water content and temperature in adjacent polluted and clean streams. The soils in the basin are predominantly sandy with the upstream section having larger fraction of coarse material. Extremely low water contents show the effect of the coarse-grained soil, hyper-arid climate and high evaporation rate. The results of the two-year monitoring at the downstream section indicate that during rain and runoff events, infiltration in the contaminated stream is considerably lower than in the clean stream. The measured water contents at the contaminated stream showed irregular patterns that can be explained by preferential flow pathways, which are known to prevail in hydrophobic soils. No consistent results were obtained in the upstream section since overland flow in the clean stream was considerably smaller compared to the contaminated stream. Nevertheless, infiltration patterns were different than in the downstream section, due to the coarse soil texture. Unsaturated hydraulic properties of clean and contaminated field samples were measured. In accordance to previous studies, it was found that oil increases the saturated hydraulic conductivity but decreases water retention.

In the two stations, the temperatures in the contaminated stream were found to be consistently higher than in the clean stream (up to 1 °C). These differences were greater in the downstream station probably due to the more severe contamination in this location. Such difference may result from the darker color of the oil-polluted soil or from lower evaporation from the contaminated soil. In other polluted sites, elevated soil temperatures were related to changes in animal behavior. This is now being studied in Evrona nature reserve in the scope of a comprehensive ecological monitoring program.

To study the optimal conditions under which oil and hydrophobicity are attenuated and to investigate the relation between the two parameters we performed an incubation experiment. During the 1.5-year incubation, we periodically measured the hydrophobicity and hydrocarbon content of 10 different treatments that included addition of combinations of water nutrients and surfactants. The results of the incubation experiment show a concomitant decrease in hydrophobicity and hydrocarbon content in soils to which nutrient, surfactants, or water to 20% saturation were added. These soils showed a reduction in their WDPT and MED values indicating hydrophobicity decrease and a decrease in TPH, in the ratio of low weight to high weight alkanes and increase in the ratios of Pr/C₁₇ and Ph/C₁₈, suggesting degradation of low-weight alkanes. At the end of the incubation experiment, the level of hydrophobicity and TPH were relatively similar in all these treatments. The rate of decrease, however, varied between treatments. The fastest degradation was observed in soil saturated to 20% treated with addition of nutrients and surfactants. The second

fastest rate was demonstrated in soil saturated to 20% to which nutrients were added. Addition of only water and surfactants showed a delayed response, while the slowest response was obtained when only water to 20% saturation was added. When water was added to 50% saturation, degradation and hydrophobicity reduction were minor and some of the hydrophobicity measures even remained unchanged. Degradation and hydrophobicity reduction in soil saturated to 20% was higher than in soil saturated to 50%. This shows that wetting the soil to 20% saturation is enough to establish sufficient conditions for bioremediation (in terms of water alone) and higher water saturation is not necessary. The 1975 soil treated with addition of nutrients showed a faster response compared to the 2014 soil. This may be the result of the local microbial community which was adapted to oil degradation (Girsowicz et al., 2018).

Overall, in the 1.5 year of the experiment the TPH of the contaminated soil decreased by 40% in the 2014 soil and by up to 82% in the 1975 soil. This shows that addition of water is sufficient to trigger substantial bioremediation which is accompanied by hydrophobicity decrease. Yet, it is important to note that the soils are still severely hydrophobic and water repellent.

Measurements of water retention in the different treatments did not show significant differences between the clean and contaminated samples, but did show that in treatments where surfactants and/or nutrients were added hysteresis was considerably lower.

Abundance of oil degraders was evaluated by qPCR and MPN, the results showed that these bacteria are found in all treatments, even in the clean soil. Surprisingly, the 1975 soils did not show higher numbers of bacteria relative to the 2014 soils, similarly no clear effect was shown by any of the treatments. The abundance of alkane monooxygenase (alkB), naphthalene dioxygenase (nahA), and phenol monooxygenase (dmpN) genes in the different treatments in the soil treated with water, nutrients, and biosurfactants did not significantly increase compared to the untreated contaminated soil at the end of incubation for 2014 soil samples. The 50% water saturation treatment exhibited the maximum numbers of copies of these genes. This may be explained by long adaptation of the functional degraders to the high water saturation conditions which induced growth after a long lag time, leading to peak number of copies at the end of the incubation experiment.

Finally, biosurfactant-producing strains were isolated and their activity was evaluated. Eight high-potential strains were isolated. All belong to the *Pseudomonas* genus, and were found to be very similar to each other in terms of 16S rRNA sequencing. When grown on hexadecane as a carbon source, the isolated strains (NS1-NS8) presented positive results in the oil spreading assay, reduced the surface tension by ~35% and presented an emulsification activity of ~45%. The results of this study indicate that six of the isolated *Pseudomonas* sp. NS1, NS2, NS3, NS5, NS6, NS8 are efficient in biosurfactant production and thus exhibit the potential to be used for oil bioremediation.

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